THE FAILURE OF A MAJOR HISTOCOMPATIBILITY ANTIGEN TO STIMULATE A THYROID ALLOGRAFT REACTION AFTER CULTURE IN OXYGEN*

By FRANCISCO G. LA ROSA and DAVID W. TALMAGE

From the Webb-Waring Lung Institute and Department of Microbiology, University of Colorado Health Sciences Center, Denver, Colorado 80262

The prolongation of allograft survival after culture in oxygen is now well established (1–5). However, to our knowledge there have been no reports of a systematic study of the histological appearance of the surviving cultured allografts during the first few weeks and months after transplantation.

We have observed that at 5 wk after transplantation into C57BL/6 recipients, cultured BALB/c thyroid grafts contained a remarkably variable degree of lymphocyte infiltration. Some of the grafts contained only a few small foci of infiltration, most contained larger foci separated by normal, uninfiltrated glandular tissue, and 20–30% were either totally infiltrated or destroyed.

The following experiments were undertaken to determine the cause of the variable infiltration. First, was it due to a variability in the graft (e.g., incomplete removal of passenger leukocytes), or to a variability in the host response to cultured grafts? Second, which antigens were responsible for the infiltration, i.e., major or minor histocompatibility antigens?

The results indicate that the variability was mostly due to the host and that the infiltration represented a response to some minor antigen(s) of the graft. We propose that this minor antigen(s) has a relatively unique capacity to induce a delayed hypersensitivity in the absence of graft leukocytes by means of indirect presentation on host macrophages. For an unknown reason major histocompatibility antigens appear to lack this capacity. However, the presence of a major antigen in a cultured graft enhanced the rejection response when the minor antigen(s) was also present.

Materials and Methods

Mice. Male BALB/cBYJ, C57BL/6, C3H, and B6.C ($H\text{-}2^d$) mice were obtained from The Jackson Laboratory, Bar Harbor, ME. BALB.B ($H\text{-}2^b$) mice were obtained from the National Jewish Hospital of Denver, Denver, CO.

Culture and Transplantation. Donor mice were killed one at a time with ether. Under the dissecting microscope the thyroid lobes were taken out immediately, put in culture medium (minimum essential medium, pH 6.9, with $NaHCO_3$ 1.1 g/liter, and penicillin and streptomycin), and trimmed free of connective tissue. The thyroids to be cultured were put in new medium (10 ml) in 60 × 12-mm plastic petri dishes (1 lobe per ml) and loaded in pressure chambers, which were rinsed with a 95% O_2–5% CO_2 mixture and pressurized with the same mixture to 23 psi (1,700 mmHg, O_2). The chambers were incubated at 37°C for 48 or 60 h. As

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the pressure chamber came to this temperature, the pressure rose to 25 psi. Thyroid lobes (cultured or uncultured) were placed under the right (or right and left) kidney capsules of each recipient under seconal anesthesia.

Assay of Thyroid Function. At 3 or 5 wk after transplantation, the recipients received 0.25 μCi carrier-free ^{125}I i.p., and 18–24 h later the mice were killed. The ^{125}I uptake by the graft was determined by placing each kidney or separated thyroid in a tube containing 1 ml of 10% formalin and counting them in a gamma counter, Beckman model 5500 (Beckman Instruments, Fullerton, CA).

Histology. After fixation, thyroids were embedded in paraffin, and three separated sections were mounted and stained with hematoxylin and eosin. An arbitrary histopathological classification was made according to the degree of lymphocytic infiltration found in the three thyroid sections: syngeneic (S), no infiltration in any of the sections; focal minus (F^-), one or more small foci of infiltration in the three sections, or a medium focus in one section balanced by another section without infiltration; focal plus (F^+), medium or large foci of infiltration in all the sections, but some areas of thyroid tissue were without damage or infiltration; general (G), lymphocytic infiltration around all the follicles, usually with severe damage in all the sections; destruction (D), all the thyroid tissue in the three sections completely destroyed. No follicles were seen.

Results

Variable Infiltration into Cultured Grafts is Largely Due to the Host. Individual thyroid lobes were cultured for 48 h and transplanted to four groups of 10 C57BL/6 mice, each according to the following protocol: group A received one BALB/c thyroid lobe under the right kidney capsule; group B received two BALB/c lobes, both under the right kidney capsule; group C received four BALB/c lobes, all under the right kidney capsule; group D received one BALB/c lobe under the right kidney capsule and one C3H lobe under the left kidney capsule.

34 d after transplantation, each recipient was given 0.25 μc ^{125}I i.p. and killed 24 h later. Individual thyroid lobes were then separated, counted in a well gamma counter, and processed for histological examination.

The results from groups A, B, and C are given in Table I and from group D in Table II. A comparison between groups B and D is given in Fig. 1. There was a marked correlation in both the ^{125}I uptake and in the degree of infiltration within the two or four thyroids from the same recipient. However, there was no evidence of interaction between the multiple lobes in the same recipient since the degree of infiltration was not affected by the number of lobes. For example, 2 of 10 single lobes were completely destroyed and in 2 of 10 receiving 4 lobes, all 4 were destroyed in each animal. None of the thyroids from recipients receiving two lobes were destroyed, but in one case both lobes were generally infiltrated. The only lobes showing no infiltration in the first three groups were two from the same recipient in group B.

The correlation between the reactions in one BALB/c and one C3H thyroid was almost as good as that between two BALB/c thyroids (Table II and Fig. 1). There was only one exception to this rule: mouse 5D exhibited large focal infiltrates in the BALB/c thyroid and total destruction of the C3H.

Infiltration in Cultured Grafts Is Due to Some Minor Antigen(s). It seemed unlikely that the correlation in the response to the BALB/c and C3H thyroids was due to a cross-reaction in the response to major histocompatibility antigens. The possibility of common minor antigens in the two donor strains was suggested. For this reason, the response of C57BL/6 (H-2^b) mice was determined to cultured and uncultured thyroid grafts from BALB/c (H-2^d), B6.C (H-2^d on a B6 background), and BALB.B (H-2^b on

TABLE I

The ^{125}I Uptake and Histology of Multiple Cultured BALB/c Thyroids Placed in the Same C57BL/6 Recipient

Mouse	^{125}I uptake (5 wk)				Histology*
	T1	T2	T3	T4	
1A	42,055	—	—	—	F^-
1B	11,705	8,155	—	—	F^+F^+
1C	12,005	7,050	7,318	14,567	$F^+F^+F^+F^+$
2A	6,740	—	—	—	F^+
2B	19,454	14,483	—	—	F^-F^-
2C	232‡				DDDD
3A	28,225	—	—	—	F^+
3B	7,879	10,814	—	—	F^+F^+
3C	7,312	7,640	6,830	10,480	$F^+F^+F^+F^+$
4A	221	—	—	—	D
4B	3,003	658	—	—	F^+F^+
4C	61‡				DDDD
5A	38,726	—	—	—	F^-
5B	16,794	17,500	—	—	F^-F^-
5C	7,313	8,749	2,891	9,068	$F^+F^+F^+F^+$
6A	13,456	—	—	—	F^+
6B	10,261	8,494	—	—	SS
6C	8,362	10,167	7,555	6,034	$F^+F^+F^+F^+$
7A	11,164	—	—	—	G
7B	16,879	15,992	—	—	F^+F^-
7C	10,252	8,715	16,086	12,552	$F^+F^-F^-F^+$
8A	14,417	—	—	—	F^+
8B	12,547	14,856	—	—	F^-F^-
8C	6,227	2,500	5,525	5,052	$F^-F^-F^-F^-$
9A	1,104	—	—	—	D
9B	15,439	9,755	—	—	F^-F^-
9C	16,801	12,998	13,629	—	F^+GF^+§
10A	3,358	—	—	—	G
10B	12,561	9,090	—	—	GG
10C	6,455	11,951	13,293	8,427	$F^+F^+F^+F^+$

* For histology classification see Materials and Methods.
‡ Total count for all four grafts.
§ No graft found, technical loss assumed.

a BALB/c background). Similarly, the response of BALB/c mice to C57BL/6 and BALB.B grafts was determined. Uncultured grafts were assayed at 3 wk and cultured grafts at 5 wk. Results from grafts cultured at 2.5 atmospheres O_2 for 48 h are given in Table III.

Approximately two-thirds of the cultured grafts (20/29) that differed from the recipient in either major antigens only, or minor antigens only, were without infiltration, compared with only 2 of 40 grafts that differed in both major and minor antigens. This suggested a synergism between major and minor antigens in the rejection process.

However, when the culture time was increased from 48 to 60 h and the responses of BALB/c and C57BL/6 mice to identical B6.C grafts were determined (Table IV), a striking difference between major and minor antigens was apparent. None of 10

TABLE II

The ^{125}I Uptake and Histology of One BALB/c and One C3H Thyroid Placed in the Same C57BL/6 Recipient

Mouse	^{125}I uptake (5 wk)		Histology*
	BALB/c thyroid	C3H thyroid	
1D	31,467	12,379	F^-F^-
2D	24,066	13,778	F^-F^-
3D	9,645	20,845	F^-F^-
4D	924	137	GD
5D	13,858	179	F^+D
6D	27,238	21,094	SF^-
7D	15,722	5,090	GG
8D	2,097	3,385	GG
9D	22,578	19,917	F^-F^-
10D	35,009	18,998	F^-F^-

* For histology classification see Materials and Methods.

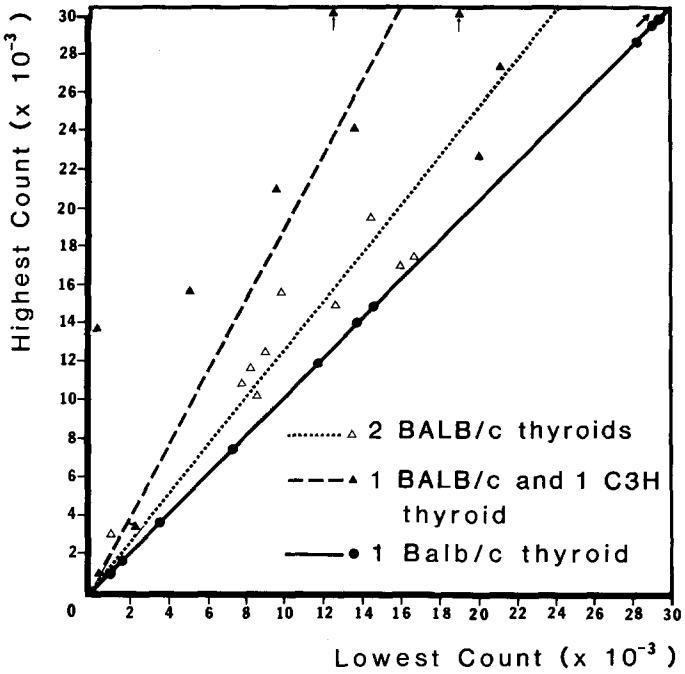

FIG. 1. A correlation between the ^{125}I uptake of two cultured thyroid lobes placed in the same C57BL/6 recipient for 5 wk. Open triangles and dotted line are two BALB/c lobes under the same kidney capsule. Closed triangles and dashes are one BALB/c and one C3H lobe under opposite kidney capsules. Variability in uptake of single BALB/c lobes is given for comparison (-●-). Data are from groups A, B, and D of Tables I and II.

grafts with major antigens only showed any infiltration, whereas 8 of 9 grafts with minor antigens only were focally infiltrated. Typical sections from these grafts are shown in Fig. 2.

TABLE III

The Degree of Infiltration in Various Grafts Cultured 48 H

	Thyroid culture (48 h)	Donor	Recipient	Antigenic differences	Number of mice	Distribution of infiltration 3 or 5 wk after grafting*					Percent S and F^-
						S‡	F^-	F^+	G	D	
1.	−	BALB/c	C57BL/6	H-2 and minor	10	0	0	0	0	10	0
2.	+	BALB/c	C57BL/6	H-2 and minor	30	1	7	14	4	4	27
3.	−	B6.C (H-2^d)	C57BL/6	H-2	16	0	0	0	1	15	0
4.	+	B6.C	C57BL/6	H-2	9	6	2	1	0	0	89
5.	−	BALB.B (H-2^b)	C57BL/6	Minor	8	0	0	1	4	3	0
6.	+	BALB.B	C57BL/6	Minor	10	6	1	1	2	0	70§
7.	−	C57BL/6	BALB/c	H-2 and minor	10	0	0	0	0	10	0
8.	+	C57BL/6	BALB/c	H-2 and minor	10	1	3	5	1	0	40
9.	−	BALB.B	BALB/c	H-2	10	1	1	1	3	4	20
10.	+	BALB.B	BALB/c	H-2	10	8	2	0	0	0	100

* Cultured grafts were assayed 5 wk after transplantation, uncultured grafts at 3 wk.
‡ For histologic classification see Materials and Methods.
§ Different from line 2 by χ^2 analysis at $P = 0.015$.

TABLE IV

The Degree of Infiltration in Grafts Cultured for 60 H

	Thyroid culture (60 h)	Donor	Recipient	Antigenic differences	Number of mice	Distribution of infiltration 3 or 5 wk after grafting*					Percent S and F^-
						S‡	F^-	F^+	G	D	
1.	−	B6.C (H-2^d)	C57BL/6	H-2	16	0	0	0	1	15	0
2.	+	B6.C	C57BL/6	H-2	10	10	0	0	0	0	100
3.	−	B6.C	BALB/c	Minor	8	0	0	0	6	2	0
4.	+	B6.C	BALB/c	Minor	9	1	4	4	0	0	55§
5.	−	BALB/c	C57BL/6	H-2 and minor	10	0	0	0	0	10	0
6.	+	BALB/c	C57BL/6	H-2 and minor	10	0	5	0	2	3	50

* Cultured grafts were assayed 5 wk after transplantation and uncultured grafts at 3 wk.
‡ For histologic classification see Materials and Methods.
§ Different from line 2 by χ^2 analysis at $P = 0.02$.

Discussion

The first group of experiments in this study were performed to determine the cause of the variability in the response of C57BL/6 mice to BALB/c thyroid allografts. Our initial assumption had been that the variability was a result of inadequate culture of the grafts, leaving a small but variable number of passenger leukocytes to stimulate a response. When extensive efforts to improve the culture condition failed to remove the variability, we tested the question directly by the experiments reported in Table I and Fig. 1. The result was a striking correlation in the degree of infiltration and the amount of residual function (iodine uptake) between the two or four grafts placed in the same individual, but no change in the range and degree of infiltration.

In earlier experiments with Lafferty et al. (1), we had shown that an uncultured graft was rejected long before a cultured graft placed in the same recipient. For this reason a variability in the effectiveness of culture might show up as differences in the degree of infiltration between multiple grafts in the same recipient. However, even if

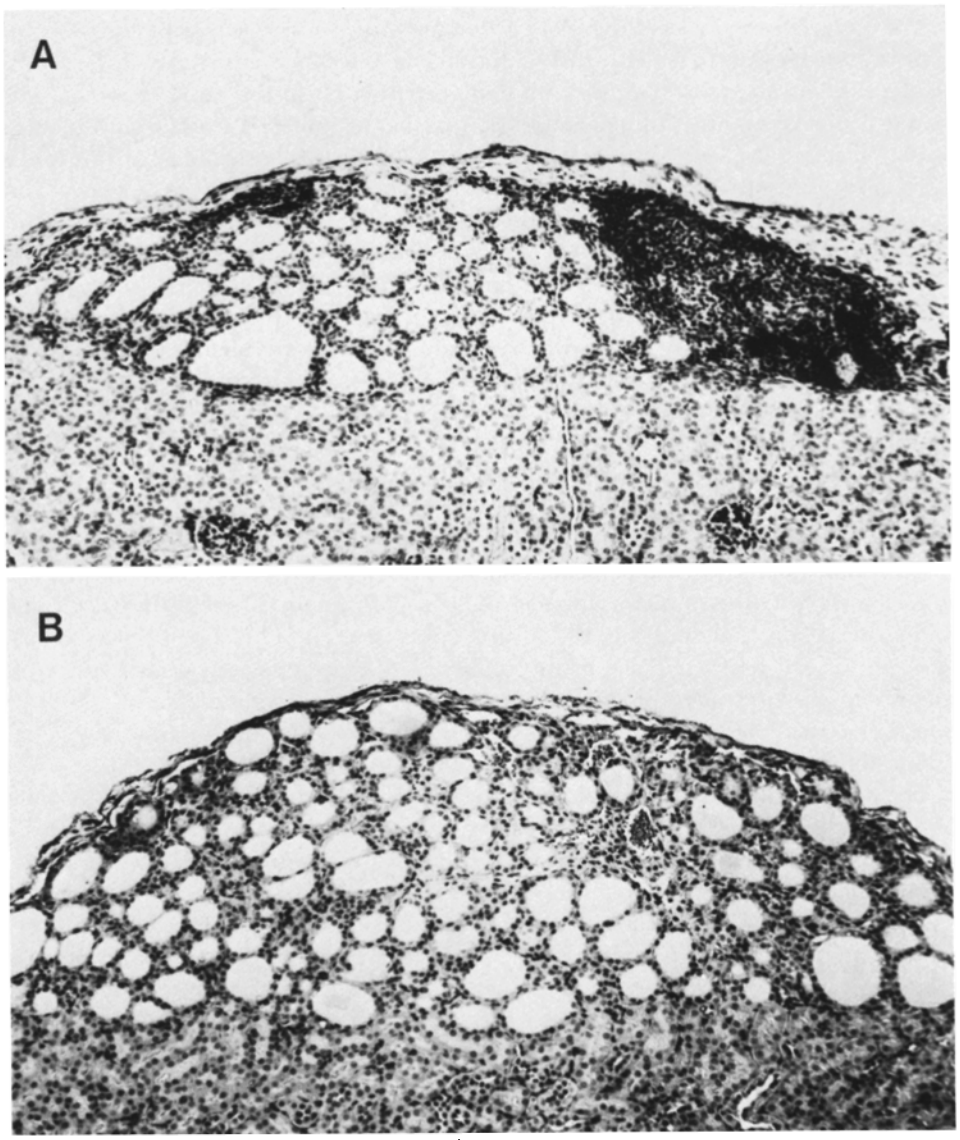

FIG. 2. Representative sections of B6.C (H-2^d) thyroid lobes cultured for 60 h and transplanted for 5 wk into (A) BALB/c recipients (minor antigens only) and (B) C57BL/6 recipients (major antigens only).

this were not true, the placement of multiple grafts in one recipient should increase the probability that one of them was inadequately cultured. Thus, even if the stimulation by residual leukocytes in one graft were quickly followed by infiltration in all the grafts, a change in the range and degree of infiltration ought to result. Since we could see neither a variability in the infiltration or function of the multiple grafts in the same recipient, nor any increase in the degree of infiltration, we concluded that the variability was probably largely a host function. This is not to say that the cultured grafts were thereby completely uniform, but only that we had to contend with a host variability of even greater dimension.

We were led to the consideration of minor antigens as a cause of the variable infiltration and thus to experiments with congenic mice by our analysis of the results of the experiments with multiple cultured grafts placed in the same recipient. The marked correlation in ^{125}I uptake and histology in two cultured grafts from the same strain placed in the same recipient (Fig. 1 and Table I) was in striking contrast to the variability in response when the grafts were placed in different recipients. This correlation was also found when one BALB/c (H-2^d) graft and one C3H (H-2^k) graft were placed in the same recipient (Fig. 1 and Table II). Since these two grafts from the BALB/c and C3H donor strains contained many common minor antigens not present in C57BL/6 mice, the hypothesis was suggested that a minor antigen(s) common to the two donor strains was responsible for the variable infiltration.

The most important and surprising finding in this study was the complete absence of infiltration in 10 thyroid allografts differing from the recipient in major histocompatibility antigens only and cultured for 60 h (Table IV). Since uncultured grafts in this combination were promptly rejected, this finding is evidence for both the adequacy of the 60-h culture and the importance of some minor antigen(s) in the production of infiltration and damage in cultured grafts.

The finding of infiltration in eight out of nine similarly cultured grafts transplanted to recipients differing in minor antigens only is confirmation of the capacity of some minor antigen(s) to induce infiltration in a cultured graft. The absence of extensive damage in such grafts and the greater infiltration and damage in grafts with both major and minor antigens suggests that the major antigens may play a role in graft rejection under these circumstances, but only after infiltration is initiated by the minor antigen(s).

The data in Table III from grafts cultured at only 48 h are less clear, and this may be an indication that this time period is slightly short of that needed to rid the grafts of leukocytes. The difference between major and minor antigens is present but not significant. This is partly the result of some infiltration in three grafts with major antigens only (possibly because of inadequate culture) and partly because of less infiltration in the grafts with minor antigens only. Nevertheless, the synergism between major and minor antigens in the production of infiltration was definite. Considering only the experiments in Table III, in which C57BL/6 mice received cultured grafts, 89% were without significant infiltration (S or F^-) with major antigens only, 70% with minor antigens only, and only 27% when both were present. The difference between the last two groups is significant by the χ^2 test at $P = 0.015$.

When the data from the two tables are pooled, 28 of 29 (97%) major antigen grafts were without significant infiltration, 12 of 19 (63%) of minor grafts, and 17 of 50 (34%) of major plus minor. These differences are significant at $P = 0.02$ or lower.

From these data we can conclude the following with decreasing certainty: (*a*) The effect of culture in eliminating the lymphocyte infiltration into grafts is greater with major antigens than with minor antigens, as evidenced by a reversal after culture in the relative importance of the two sets of antigens. (*b*) The minor antigens have a greater effect than major antigens in producing infiltration in cultured grafts when either set is present alone. (*c*) The presence of major antigens increases the infiltration and destruction caused by minor antigens. (*d*) Major antigens probably do not produce any infiltration in an adequately cultured graft when present alone.

Our interpretation of the findings is as follows: The culture in oxygen removes

passenger leukocytes and in particular the Ia^+ macrophages required for the activation of helper T cells. An alternative to direct activation of T cells by graft macrophages involves the shedding of graft antigen and its pick-up and presentation by host macrophages. The T lymphocyte response to this antigen amounts to a type of delayed hypersensitivity. Not all proteins are equally effective in inducing this type of delayed hypersensitivity response. Our results suggest that major antigens are ineffective and that some minor antigen(s) is relatively more effective.

According to this interpretation, the variability in the infiltration in different recipients is due to a variable balance between the delayed hypersensitivity, which develops to some minor antigen(s), and a variety of suppressor mechanisms known to affect this kind of immune response (6–8).

The finding that culture has more effect on grafts containing major antigens than those containing minor antigens only is in accord with data reported by Naji et al. (9). In their experiments involving the transplantation of parathyroids in rats, culture for 26 d in 95% O_2 reduced the survival of Lewis strain grafts to Fisher strain rats (minor antigens only), but increased the survival of ACI grafts (major plus minor antigens). However, the survival of cultured ACI grafts was still lower than that of cultured Lewis grafts. Thus, their data are also in accord with our conclusion that major antigens enhance the rejection response to minor antigens in a cultured graft.

In a subsequent paper, Silvers et al. (10) made the surprising prediction that cultured "grafts might be more likely to survive in major histocompatibility complex (MHC) incompatible hosts." This suggestion was based on the inability of B6 mice made tolerant of A × B6 lymphoid cells after γ radiation to reject A strain skin grafts that had been maintained for 100 d on intermediate, unresponsive B6 hosts. They proposed that a major antigenic difference should inhibit the response to a minor antigen because of a failure of T cells recognizing antigen on host macrophages to react with the same antigen on an MHC-incompatible graft. While we agree with this interpretation of their data, it is necessary to point out that their B6 recipients were tolerant of the MHC antigens of the graft and thus could not respond to them.

We are proposing that in the inflammatory environment of a relatively nondestructive delayed hypersensitivity response to minor antigens, a direct and more rapidly destructive response to major antigens can occur in normal recipients in the absence of passenger leukocytes. It is tempting to speculate that this response may be an interleukin 2-dependent cytotoxic T cell response.

Summary

The lymphocytic infiltration found in multiple cultured BALB/c thyroids placed in the same C57BL/6 recipient was found to be highly correlated and the high variability between animals was not influenced by the number of lobes. It was concluded that the variable infiltration was largely due to host factors. Because a similar correlation was found between BALB/c and C3H grafts, the response to a minor antigen common to these two strains was suggested as a cause of the infiltration. When the response of C57BL/6 mice to cultured B6.C ($H\text{-}2^d$) and BALB.B ($H\text{-}2^b$) grafts was compared, a synergism between major and minor antigens was suggested. However, when the time of culture was increased from 48 to 60 h and the response of BALB/c and C57BL/6 mice were compared with identically cultured B6.C ($H\text{-}2^d$) grafts, a striking difference between major and minor antigens was observed. None of

10 such grafts in C57BL/6 recipients (major antigens only) showed any infiltration, whereas 8 out of the 9 grafts in BALB/c recipients (minor antigens only) were infiltrated.

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