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Immunological Disease Induced by Injecting F1 Lymphoid Cells into Certain Parental Strains

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Summary. Injection of neonatal Balb/c or C57Bl/6 mice with (C57Bl/6 × Balb/c)F1 lymphoid cells leads to transient chimerism and runting, and to splenomegaly, deficient T-cell function and a gradual replacement of lymphoid organs with abnormal reticular cells. Activated MuLV can be isolated from such mice. It is proposed that either graft-versus-host or host-versus-graft allogeneic reactivity activates endogenous MuLV virus, which then causes functional and morphological abnormalities in the lymphoid organs.

INTRODUCTION

Classical tolerance to transplantation antigens can be induced by the injection of F1 lymphoid cells into neonatal parental strain mice (Billingham, Brent and Medawar, 1956). Many of the original experiments used the A and CBA strains, where the injection of (A × CBA)F1 cells into either A strain or CBA strain neonates is followed by a permanent acceptance of skin grafts of the opposite parental strain and a readily demonstrable chimerism with F1 cells (Silobrcic, 1971). In fully tolerant mice of these strains there appears to be a lack of antigen-reactive cells for parental antigens in the periphery, as shown by negative cytotoxic tests or mixed lymphocyte reactions (Beverley, Brent, Brooks, Medawar and Simpson, 1973).

When (C57Bl/6 × Balb/c)F1 cells are injected into either C57Bl/6 or Balb/c neonates, abnormalities characterized by runting and splenomegaly occur. In this paper we attempt to explore the abnormalities of these mice by examining their growth rates, the survival of allografted skin, chimerism, graft-versus-host potential, their ability to respond *in vitro* to alloantigens and mitogens and histopathological changes in their organs. Preliminary virus isolation studies have been made, and attempts have been made to passage the abnormalities with spleen cell suspensions.

MATERIALS AND METHODS

Mice

Adult male (C57Bl/6 × Balb/c)F1 mice and pregnant Balb/c and C57Bl/6 mice were

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obtained from the Animal Division at NIH, Bethesda, Maryland. Injections of F1 cells into parental strain neonates were made within 24 hours of birth. Either spleen or bone marrow cell suspensions from F1 mice were made in balanced salt solution (BSS, Media Section, NIH) and 25 or 50×10^6 cells were given in 0·1 ml i.p. to the neonates. Some litters were left uninjected, as age-matched controls.

Skin grafting

This was carried out according to the method of Billingham and Medawar (1951).

Serum allotype

This was detected by double diffusion in gel (Ouchterlony) using anti-Ig1a and anti-Ig1b reagents kindly supplied by Dr L. A. Herzenberg.

Graft versus host assays

These were carried out according to the method of Cantor and Asofsky (1970).

Cytotoxic cell assays

These were done by the method of Canty and Wunderlich (1970).

In vitro *sensitization*

Sensitization against alloantigens was carried out by the method of Wunderlich and Canty (1970) and assay for cytotoxic cells by the method of Canty and Wunderlich (1970).

Mixed lymphocyte reaction and phytomitogen stimulation

This was carried out by the method of Mosier (1973).

Tissue fixation for histology

Tissues were fixed in FAA prior to processing by the histology section of NCI. Sections were cut at 4 μm and stained with haematoxylin and eosin and with methyl green–pyronin.

Virus isolation

The XC test for infectious murine leukaemia virus (MuLV) was carried out as described by Rowe, Pugh and Hartley (1971). All cultures were grown in MEM with four times the usual concentration of vitamins and essential amino acids and 10 per cent untreated foetal calf serum. Trypsinized cells from primary NIH Swiss or Balb/c embryo cell cultures were seeded at a concentration of 4×10^5/50 mm Petri dish (Falcon plastics, Los Angeles, California). The next day the monolayers were treated with DEAE–dextran (25 μg/ml for 1 hour at 37°C in medium without serum), washed once and inoculated with 1×10^6 or 10×10^6 viable spleen cells in 4 ml of medium. Four days after inoculation the medium was changed and 7 days after inoculation the cultures were exposed to u.v. light (9 seconds at a distance of 27 cm from two General Electric germicidal bulbs), causing death of the embryo cells, but not the virus (Rowe *et al.*, 1971). After irradiation, 1×10^6 XC cells were added per dish. Three days later the monolayers were fixed with methanol and stained with Giemsa. When MuLV is present in the embryo cells, the XC cells form syncytia resulting in the formation of macroscopically visible plaques. The XC line itself, which is derived from a Rous sarcoma-induced rat tumour, does not form syncytia. Plaques

were identified by scanty growth of XC cells in areas of syncytium formation and were distinguished from nonspecific defects in the cell layer by the presence of at least three syncytia.

RESULTS

Table 1 shows the number of Balb/c and C57Bl/6 neonates injected with F1 cells, and the dose and source of F1 lymphoid cells used. The eighty-four Balb/c mice were born in fourteen litters, of which eleven litters were injected, whilst the 27 C57Bl/6 mice were born in five litters of which four litters were injected. The progeny of uninjected litters were kept as age-matched controls.

TABLE 1

EXPERIMENTAL DESIGN (C57Bl/6 × Balb/c) F1→ NEONATAL Balb/c AND C57Bl/6 MICE

Recipients	Numbers and source of F1 lymphoid cells				Total number injected
	No cells	50×10^6 spleen	50×10^6 bone marrow	20×10^6 spleen	
Balb/c (male)	11	16	1	7	
Balb/c (female)	11	23	6	9	
Total Balb/c	22	39	7	16	62
C57Bl/6 (male)	2	15	—	—	
C57Bl/6 (female)	5	5	—	—	
Total C57Bl/6	7	20			20

GROWTH CURVES

The Balb/c and C57Bl/6 mice were weighed weekly from birth for 11 weeks. Two periods of runting were seen in the Balb/c recipients, one very early, at 2 weeks, involving two of the litters which had received 50×10^6 spleen cells. All progeny of these two litters died. There was a second, more characteristic period of runting between 5 and 7 weeks of age, during which period mice lost weight, developed ruffled fur and looked ill. A few died at this time, but the majority apparently recovered, at least judged by weight gain and appearance. Growth curves illustrating this second period of runting are shown in Fig. 1. Fig. 1a shows the growth curves of nine recipients of 25×10^6 F1 spleen cells: the progeny of two litters. There were four males and five females. Two females died, one at 4 weeks and one at 7 weeks. Two males were killed at the peak of runting, at 6 weeks, for cytotoxicity testing. Fig 1b shows the normal growth curves of an uninjected litter, consisting of four males and four females. Runting occurred in forty-nine of fifty-five Balb/c mice given F1 spleen cells at either dose, in two of the seven Balb/c recipients of F1 BM cells, and ten of the twenty C57Bl/6 recipients of F1 spleen cells. The C57Bl/6 mice which runted all did so when 5–7 weeks of age. These figures are broken down according to sex in Table 2.

SPLENOMEGALY

Mice runting at either 2 or 5–7 weeks, when examined post-mortem all had enlarged

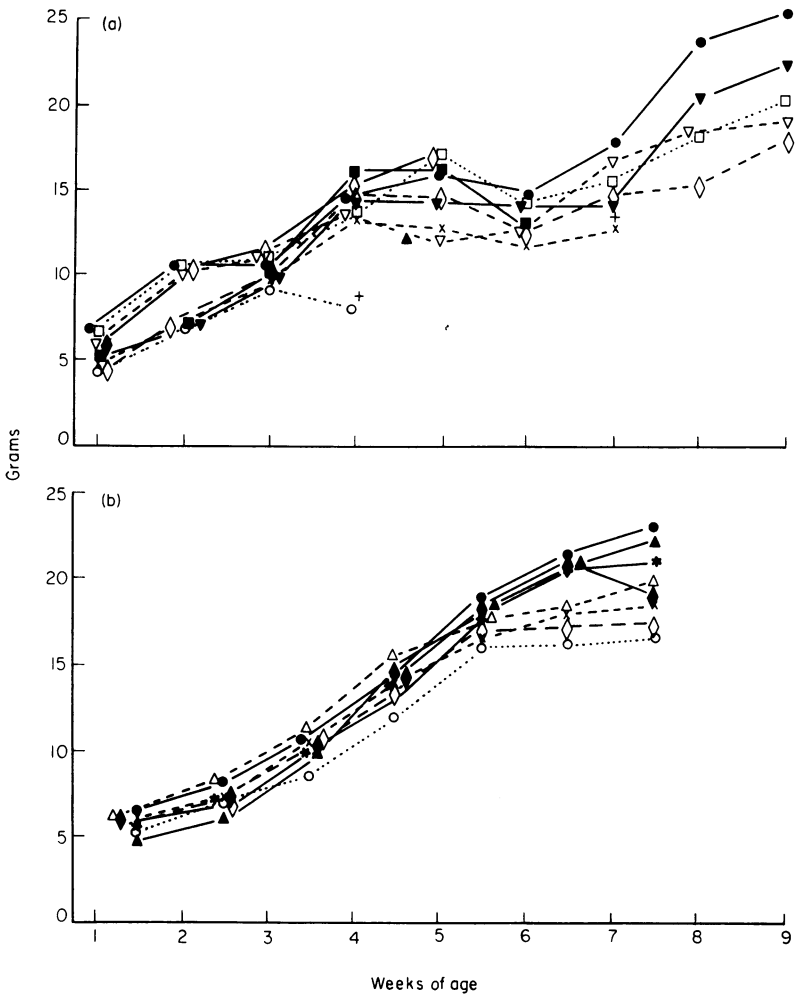

FIG. 1. Growth curves of Balb/c recipients of (C57Bl/6 × Balb/c)F1 spleen cells at birth (a) and of control uninjected Balb/c mice (b). Each line represents the growth of an individual mouse. (●), (▲), (*), (◆), (■) and (▼) are males, (○), (◇), (×), (△), (□) and (▽), are females.

TABLE 2

INCIDENCE OF RUNTING IN Balb/c AND C57Bl/6 RECIPIENTS OF (C57Bl/6 × Balb/c)F1 CELLS AT BIRTH

Recipients	Number and source of injected cells	
	25 or 50 × 10^6 F1 spleen	50 × 10^6 F1 bone marrow
Balb/c (male)	19/23	0/1
Balb/c (female)	30/32	2/6
C57Bl/6 (male)	7/15	—
C57Bl/6 (female)	3/5	—

spleens. This was a feature of all the injected mice, either Balb/c or C57Bl/6, killed at any age, whether or not they had shown runting. In many cases the spleen was grossly enlarged, filling the entire abdominal cavity. Such spleens would yield up to 1×10^9 cells (compared to $1–2 \times 10^8$ for normal spleens) when cell suspensions were made of them. An example of gross enlargement is shown in Fig. 2, a photograph taken of a C57Bl/6 mouse at 5 months of age. Enlargement of all lymph nodes was also apparent.

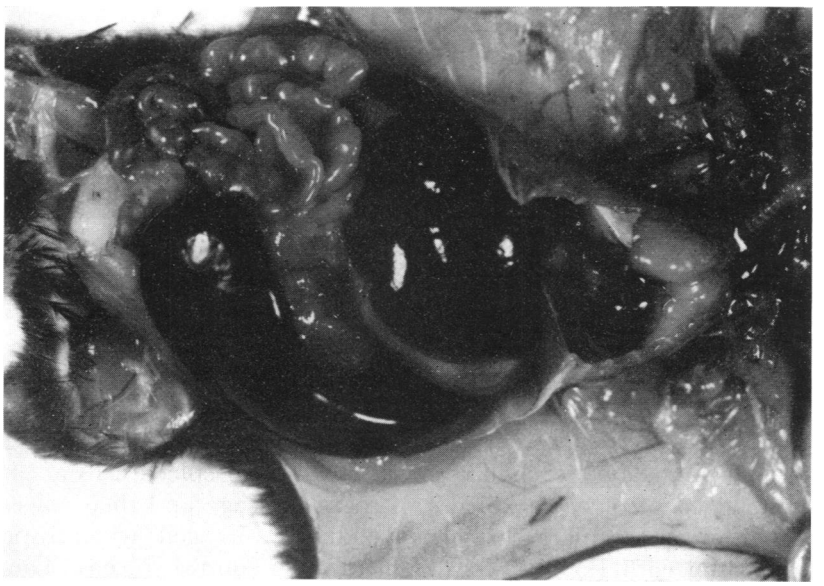

FIG. 2. Gross pathology of a 5-month-old C57Bl/6 recipient of (C57Bl/6 × Balb/c)F1 spleen cells. Note grossly enlarged spleen and lymph nodes. The mass in the anterior mediastinum was shown, on histological sectioning, to be enlarged parathymic lymph nodes. The thymus lay beneath them, and was normal histologically (see Fig. 10).

CYTOTOXICITY TESTING

To examine the possibility that the runting and splenomegaly observed might be due to cytotoxic cells, either of parental cells, directed against antigens present on the injected F1 cells (host-versus-graft), or of F1 cells directed against an unknown antigen present in the parental recipient (graft-versus-host), direct cytotoxicity testing was carried out. Two experiments were performed: the first using spleens from Balb/c mice found runted and moribund at 2 weeks of age, and the second using spleens from two Balb/c mice at 5 weeks old, which had not yet runted, two Balb/c mice at 6 weeks, showing runting and two Balb/c mice at 8 weeks, having apparently recovered from runting. Spleen cell suspensions were made from these mice, washed once and suspended at 8×10^6/ml in Eagle's Minimal Essential medium with 10 per cent FCS, and added in 1-ml aliquots to 5×10^5 ^{51}Cr-labelled target cells. Target cells used were EL-4 (of C57Bl/6, H-2^b, origin) and LSTRA (of Balb/c, H-2^d, origin). Spleen cells and target cells were incubated together for 4 hours on a rocking platform (Canty and Wunderlich, 1970) and the percentage of ^{51}Cr release subsequently measured. No specific cytotoxic activity was found against either target in either experiment (Table 3).

TABLE 3

DIRECT CYTOTOXICITY MEASUREMENTS OF SPLEENS FROM Balb/c MICE INJECTED NEONATALLY WITH (C57Bl/6 × Balb/c)F1 CELLS

Experiment number	Attacking cells	Target cells* EL-4 Percentage ^{51}Cr release	EL-4 Specific† activity	LSTRA Percentage ^{51}Cr release	LSTRA Specific† activity
1	Normal Balb/c	6·63		8·81	
	Normal C57Bl/6	6·19		9·96	
	Injected Balb/c (2 wks)	6·35	−0·28 ± 0·25	8·90	−1·06 ± 0·23
2	Normal Balb/c	6·26		5·44	
	Injected Balb/c (5 wks)	6·81	0·56 ± 0·14	5·59	0·15 ± 0·19
	Injected Balb/c (6 wks)	5·96	−0·30 ± 0·18	5·48	0·04 ± 0·19
	Injected Balb/c (8 wks)	5·98	−0·27 ± 0·14	5·53	0·09 ± 0·18

* 'Background' ^{51}Cr release of target cells incubated with medium alone. Experiment 1: EL4 6·54; LSTRA 10·89. Experiment 2: EL4 6·79; LSTRA 5·69.

† Specific activity = [(^{51}Cr release by experimental cells − ^{51}Cr release by normal cells)/(^{51}Cr release by 4 times frozen and thawed target cells)] × 100. The figure shown is the activity at an attacker: target cell ratio of 16:1. The number of ^{51}Cr-labelled target cells = 5×10^5.

GRAFT-VERSUS-HOST POTENTIAL OF SPLEEN CELLS FROM INJECTED MICE

Two female Balb/c mice which had received 50×10^6 F1 spleen cells at birth and two female age-matched controls were killed at 6 months of age and their spleens removed. The two injected mice had enlarged spleens, yielding 1×10^9 cells when suspensions were made, in comparison with $1{\cdot}5 \times 10^8$ cells from the two control spleens. The spleen cells were made up at 10^8/ml in BSS and 0·1 ml (1×10^7 cells), injected i.p. into neonates of three (C57Bl/6 × Balb/c)F1 litters. In each litter two mice were left as uninjected controls, two or three given control cells and two or three given cells from the Balb/c injected mice. At 9 days of age the F1 recipients were killed, their body weights and spleen weights measured, and the splenic index calculated for each cell suspensions injected (Cantor and Asofsky, 1970). The normal control spleen cells gave an index of 2·92 ± 0·09, indicating

TABLE 4

GRAFT-VERSUS HOST POTENTIAL OF SPLEEN CELLS FROM NORMAL Balb/c AND Balb/c INJECTED NEONATALLY WITH (C57Bl/6 × Balb/c)F1 CELLS

	Splenic index* Litter 1	Litter 2	Litter 3	Average
Normal Balb/c Spleen cells	2·77	2·69	3·09	
	2·59	3·04	3·05	2·92 ± 0·09
		3·22		
Spleen cells from injected Balb/c	1·48	1·24	1·22	
	1·19	1·14	1·15	1·26 ± 0·05
			1·41	

* Splenic index = (spleen: body weight of injected F1)/(spleen: body-weight of uninjected F1).

graft-versus-host activity, whereas the spleen cells from injected Balb/c mice gave an index of $1{\cdot}26 \pm 0{\cdot}05$, indicating no significant graft-versus-host activity (Table 4).

CHIMERISM

B cells from Balb/c mice make immunoglobulin of Ig1a allotype, whereas B cells from C57Bl/6 mice make immunoglobulin of Ig1b allotype. A population of F1 cells will contain some B cells which make immunoglobulin of Ig1a allotype and some make Ig1b allotype. Therefore, a Balb/c mouse injected with (C57Bl/6 × Balb/c)F1 cells will have some immunoglobulin of Ig1b type only if the F1 cells have established themselves in the recipient, i.e. the recipient is chimeric with respect to the injected F1 cells. Likewise a C57Bl/6 mouse will have Ig1a allotype present only if the F1 cells injected have made it a chimera. By testing for Ig1b in Balb/c recipients of F1 cells and for Ig1a in C57Bl/6 recipients of F1 cells, the presence of chimerism can be established. The Balb/c recipients of F1 cells were bled on three occasions at 3, 9 and 10 months of age, and their serum tested for Ig1b allotype. The C57Bl/6 recipients of F1 cells at birth were bled and tested for Ig1a allotype twice—once at 7 weeks of age and again at 5 months of age. The results are shown in Table 5, with a further analysis of the Balb/c chimeras in Table 6. When first

TABLE 5

TEST FOR CHIMERISM: PRESENCE OF Ig1b OR Ig1a ALLOTYPE IN Balb/c AND C57Bl/6 RECIPIENTS OF (C57Bl/6 × Balb/c)F1 CELLS AT BIRTH

	Balb/c tested at:			C57Bl/6 tested at:	
	3 months	9 months	10 months	7 weeks	5 months
Ig1b	13/34	1/20	0/7		
Ig1a				7/8	0/5

TABLE 6

Balb/c MICE INJECTED AT BIRTH WITH (C57Bl/6 × Balb/c)F1 CELLS TESTED AT 3 MONTHS OF AGE FOR Ig1b ALLOTYPE

Number and source of F1 cell inoculum		
50×10^6 (spleen)	25×10^6 (spleen)	50×10^6 (bone marrow)
11/18*	1/10*	1/6*

* Number positive for Ig1b allotype/number tested.

tested at 3 months, almost 40 per cent of the Balb/c recipients were chimeras by this criterion and this proportion diminished with age. Of those positive when first tested the majority had received the highest number of F1 spleen cells: only two of sixteen mice tested which had received the lower dose of F1 cells, showed Ig1b allotype. Similarly, the majority of C57Bl/6 mice tested at 7 weeks showed Ig1a allotype but when tested at 5 months, none of the five remaining mice were positive.

Skin grafting has been used to indicate tolerance to alloantigens and hence chimerism. For example, indefinite survival of A skin on CBA recipients of (CBA × A)F1 cells at

birth has been extensively reported (Billingham *et al.*, 1956). For the C57Bl/6–Balb/c combination the picture is complicated by the presence of different tissue-specific allelic products (SK) on the skin of C57Bl/6 and Balb/c mice (Lance, Boyse, Cooper and Carswell, 1971). Since parental recipients of F1 lymphoid cells are not exposed to skin-specific antigens at the time of neonatal injection, they should not become fully tolerant of skin grafts of the opposite parent, although some MST prolongation is expected (Lance *et al.*, 1971).

In these experiments twenty-eight Balb/c recipients were grafted with C57Bl/6 skin at the age of 3 months. Six age-matched controls rejected the C57Bl/6 skin at 9 days post-grafting, but there was a wide scatter of survival of C57Bl/6 skin on injected recipients, ranging from 9 days to more than 3 months. The results are shown in Table 7. Of the

TABLE 7

SURVIVAL OF C57Bl/6 GRAFTS ON Balb/c MICE INJECTED WITH (C57Bl/6 × Balb/c)F1 CELLS AT BIRTH (TWENTY-EIGHT MICE GRAFTED)

Days post-grafting	9	12	16	18	20	28	90
Number of grafts surviving	28	13	9	8	5	4*	1

* Three mice with grafts intact killed twenty-eight days post-grafting for other tests.

C57Bl/6 recipients of F1 cells at birth, eight were grafted with Balb/c skin at 2 months of age. Age-matched controls had rejected Balb/c skin by 9 days. There was a slight prolongation of Balb/c skin survival in F1 cell injected mice: three grafts were rejected by day 11; a further 4 by day 15; and only one remained at day 28, when the bearer was killed for further tests. From these results, however, it is quite clear that prolonged survival of skin bearing the other parental antigens (including SK) is not a prominent feature of Balb/c or C57Bl/6 mice receiving F1 cells at birth.

In vitro SENSITIZATION TO ALLOANTIGENS AND MITOGEN STIMULATION

Having failed to demonstrate cytotoxic cells to alloantigens in the spleens of neonatal Balb/c recipients of F1 cells, we attempted to induce cytotoxic responses *in vitro* to alloantigens and to measure proliferative responses to mitogens. Spleens were taken from recipient mice and from age-matched controls, and placed in culture with irradiated allogeneic cells as a source of antigen. Aliquots of the same cell suspensions were placed in culture with mitogens. Using the same responding cell suspensions, cultures were set up to generate cytotoxic cells (Mishell and Dutton, 1967; Wunderlich and Canty, 1970) and after 5 days in culture the cells were assayed against appropriate ^{51}Cr-labelled target cells. Cultures set up to measure MLR and mitogen responses were pulsed with [^{3}H]thymidine at 56 hours and harvested at 72 hours.

The results are shown in Tables 8 and 9. In the experiments in which C57Bl/6 mice were used as responders (Table 8) the injected mice were divided into groups according to whether they had been grafted or not, and age of recipients, either 9 weeks or 5 months. In the younger injected mice the response to Balb/c alloantigens and the T-cell mitogens PHA and Con A was diminished, whether the results were analysed by E:C cpm ratios

TABLE 8

ABILITY OF SPLEEN CELLS* FROM NORMAL AND INJECTED C57Bl/6 MICE TO RESPOND TO ALLOANTIGENS AND MITOGENS *in vitro*

Responding cell	Antigen/mitogen	cpm ± s.e.¶	E:C	E−C	Cytotoxicity** against Balb/c
Normal C57Bl/6†	C57Bl/6X§	1821 ± 142			
	(C57Bl/6 × Balb/c)F1X	3545 ± 199	1·95	1724	
	—	1234 ± 63			9·55 ± 0·85
	PHA (1 μg/ml)	65535 ± 3439	53·10	64301	
	Con A (2 μg/ml)	120733 ± 4724	97·83	119499	
Injected C57Bl/6 not grafted†	C57Bl/6X	1540 ± 71			
	(C57Bl/6 × Balb/c)F1X	2138 ± 131	1·38	598	
	—	1007 ± 79			−0·57 ± 0·43
	PHA (1 μg/ml)	2270 ± 89	2·25	1263	
	Con A (2 μg/ml)	3141 ± 182	3·12	2134	
Injected C57Bl/6 graft rejected†	C57Bl/6X	3078 ± 343			
	(C57Bl/6 × Balb/c)F1X	4456 ± 235	1·44	1378	
	—	1055 ± 204			0·22 ± 0·10
	PHA (1 μg/ml)	6662 ± 377	6·30	5607	
	Con A (2 μg/ml)	39759 ± 3813	37·69	38704	
Injected C57Bl/6 graft rejected‡	C57Bl/6X	16409 ± 1439			
	(C57Bl/6 × Balb/c)F1X	33138 ± 3919	2·02	16729	
	—	47769 ± 2233			−1·23 ± 0·14
	PHA (1 μg/ml)	304763 ± 14725	6·38	256994	
	Con A (2 μg/ml)	337330 ± 6600	7·06	289561	

* Pools of spleen cells from two mice in each group were used.
† At 9 weeks of age.
‡ At 5 months of age.
§ Target cells for MLR were inactivated by 2000 R irradiation (×).
¶ Arithmetic mean ± s.e.m. of four replicate cultures.
** Specific activity = [(^{51}Cr release by experimental cells − ^{51}Cr release by normal cells)/(^{51}Cr release by 4 times frozen and thawed target cells)] × 100. The figure shown is the activity at an attacker:target cell ratio of 2:1. The number of ^{51}Cr-labelled target cells = 5×10^5.

TABLE 9

ABILITY OF SPLEEN CELLS* FROM NORMAL AND INJECTED Balb/c MICE TO RESPOND TO ALLOANTIGENS AND MITOGENS *in vitro*

Responding cells	Antigen/mitogen	cpm ± s.e.¶	E:C	E−C	Cytotoxicity**	
					C57Bl/6	CBA
Normal Balb/c†	Balb/cX§	894 ± 114				
	(C57Bl/6 × Balb/c)F1X	1557 ± 608	1·74	663		
	C57Bl/6X	4571 ± 937	5·11	3677		
	CBAX	2115 ± 195	2·37	1221	18·34 ± 1·47	8·15 ± 0·14
	—	4883 ± 378				
	PHA	310180 ± 15617	63·52	305297		
	Con A	269218 ± 6208	55·13	264335		
Injected Balb/c†‡	Balb/cX	16591 ± 1680				
	(C57Bl/6 × Balb/c)F1X	20175 ± 1563	1·22	3584		
	C57Bl/6X	25301 ± 4840	1·52	8710		
	CBAX	23104 ± 1797	1·39	6513	0·05 ± 0·08	1·82 ± 0·29
	—	33340 ± 3492				
	PHA	127923 ± 21097	3·84	94583		
	Con A	122519 ± 8260	3·67	89179		

* Pools of spleen cells from two mice in each group.
† At 10 months of age.
‡ Grafted at 3 months, rejected grafts.
§ Target cells for MLR were inactivated by 2000 R irradiation (×).
¶ Arithmetic mean ± standard error of the mean of four replicate cultures.
** Specific activity = [(^{51}Cr release by experimental cells − ^{51}Cr release by normal cells)/(^{51}Cr release by 4 times frozen and thawed target cells)] × 100. The figure shown is the activity at an attacker:target cell ratio of 2:1. The number of ^{51}Cr-labelled target cells = 5×10^5.

or E – C incremental cpm. A striking feature of the older group of mice was the high rate of thymidine incorporation in the absence of specific stimulation (= background) (also see Table 9) which made it difficult to interpret the data, although by examining E/C it appeared that the response to Balb/c antigens was not abnormal, whilst the response to PHA and Con A was diminished. However, in none of the three injected groups was there any development of functional cytotoxic cells against Balb/c alloantigens. Table 9 shows the results of an experiment in which 10-month-old control and injected Balb/c mice were used. Their responses to C57Bl/6 and a third party alloantigen (CBA) were examined and by all criteria the spleen cells from injected mice had deficient T-cell function. Whilst the cells from the normal mice made good responses to both C57Bl/6 and CBA antigens (E:C and E – C in the MLR, and good cytotoxic cell responses), and the expected Con A and PHA responses, cells from injected mice had high background counts, low ratios and incremental counts to C57Bl/6, CBA and both mitogens, and their ability to make a cytotoxic response was completely abrogated in the case of C57Bl/6, and reduced to very low levels in the case of CBA. Another experiment (not shown) using Balb/c mice, both normal and injected, at 4 months of age, showed essentially the same results. In this experiment, the injected mice had been grafted 1 month previously, one group had rejected the grafts and the other had retained them, but both, in comparison with the controls, showed high background cpm, much lower than normal incremental cpm following stimulation with C57Bl/6 cells or mitogens (PHA and Con A) and no development of cytotoxic cells to C57Bl/6 antigens.

PATHOLOGY AND HISTOPATHOLOGY

Mice killed at various times during the course of the above experiments had tissues examined and removed for histopathological examination. In addition to the large spleens, already noted, enlargement of lymph nodes was a feature, especially in mice over 6 months of age, and particularly noticeable in the mediastinal and parathymic lymph nodes. These latter were frequently so large that they completely obscured the thymus, and it was difficult, macroscopically, to decide whether the enlarged organ was thymus or parathymic lymph nodes. In some mice the liver was enlarged, and in some the lungs were patchily spotted, both under the pleura and within the parenchyma. No gross abnormality of the kidneys was seen.

Histologically, there appeared to be three progressive stages. The first, which was seen in two 6-month-old Balb/c recipients of 50×10^6 F1 bone marrow (one mouse) and 50×10^6 F1 spleen cells (one mouse) showed active germinal centres in spleen and in lymph node cortex, many mitoses in the thymus-dependent area of spleen and large numbers of plasma cells in the medullary cords of lymph nodes. Depletion of small lymphocytes was not apparent at this stage. The thymus appeared to be normal. The second stage, which was seen in a 6-month-old Balb/c recipient of 50×10^6 F1 spleen cells, and three 2-month-old C57Bl/6 recipients of 50×10^6 F1 spleen cells, showed a continued increase in the number of germinal centres in spleen (Fig. 3) and lymph node cortex, but in addition the thymus-dependent areas of spleen and lymph node were relatively depleted of small lymphocytes (Fig. 3), and contained many large pyroninophilic cells (Fig. 4), many of which were in mitosis. The periarteriolar regions of spleen also contained some plasma cells (Fig. 4). Increased numbers of reticulum cells were also apparent in the paracortices of lymph nodes of mice at this second stage, and the medullary cords were packed with plasma

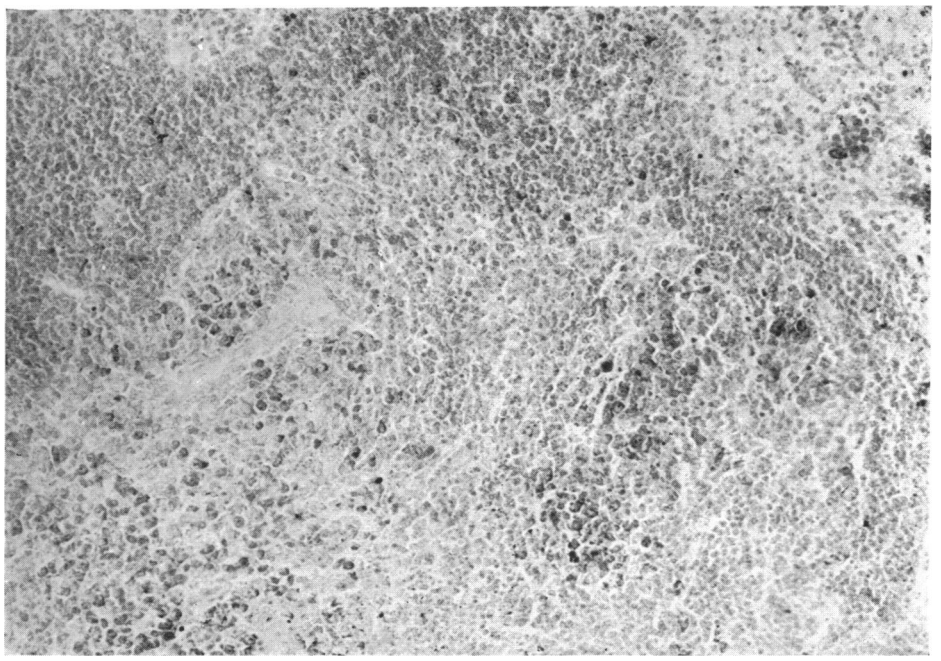

FIG. 3. Spleen from a 2-month-old C57Bl/6 recipient of (C57Bl/6 × Balb/c)F1 cells. Stage 2 features (see text). Germinal centre formation, periarteriolar region shows some depletion of small lymphocytes, and the appearance of pyroninophilic cells. (Methyl Green–Pyronin; magnification × 200.)

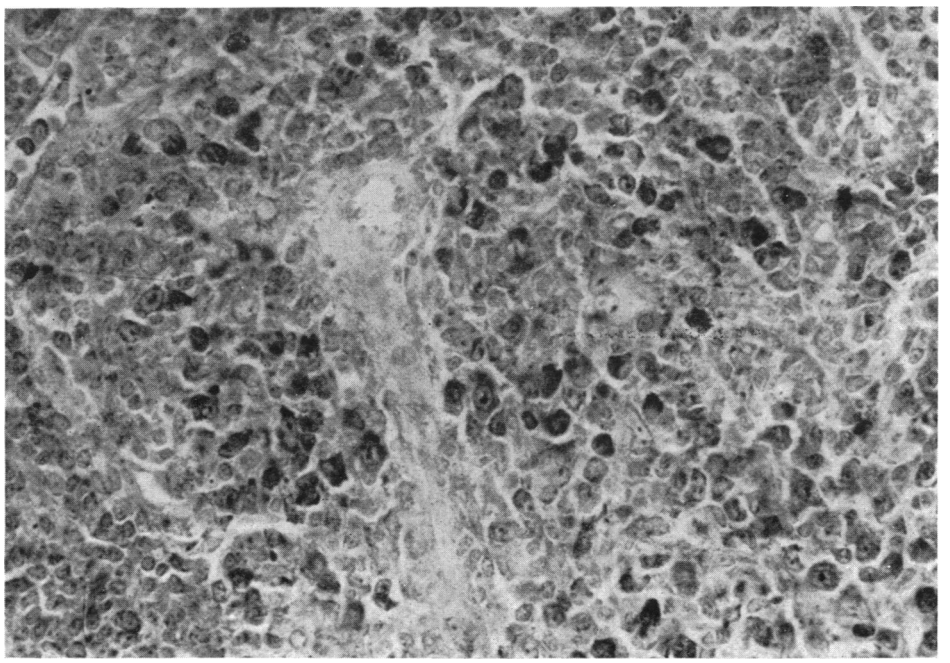

FIG. 4. Spleen from the same mouse as Fig. 3. Pyroninophilic cells in periarteriolar (thymus-dependent) region. (Methyl Green–Pyronin; magnification × 520.)

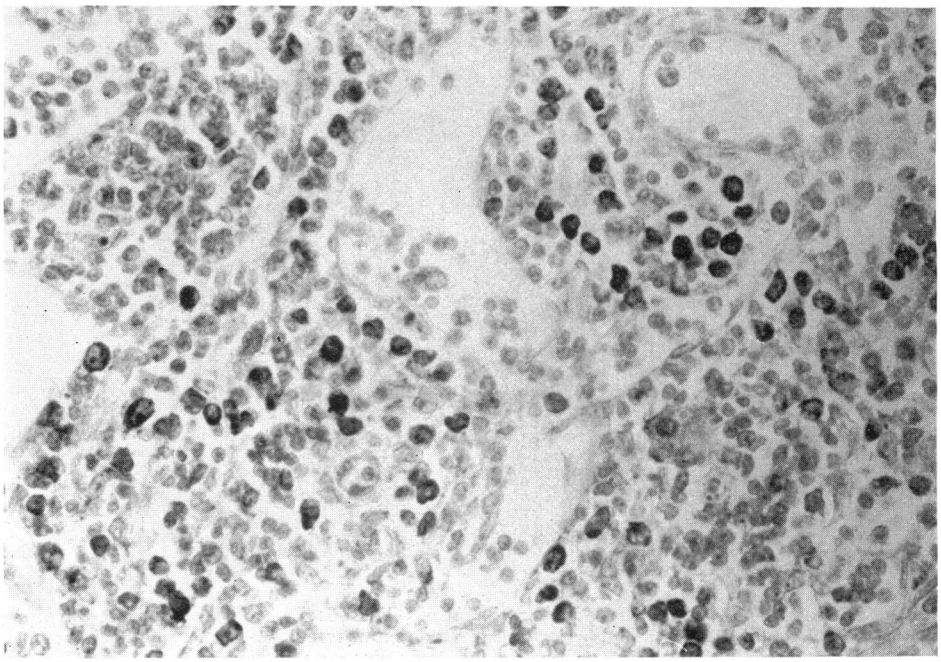

FIG. 5. Lymph node from a 2-month-old C57Bl/6 recipient of (C57Bl/6 × Balb/c)F1 cells with stage 2 features. Note medullary cords containing blast cells and plasma cells. (Methyl Green–Pyronin; magnification × 520.)

cells and blast cells (Fig. 5). The thymus was either normal or somewhat atrophied. Abnormal cellular infiltrates were also seen in the lungs of all mice at this stage and in two of the four livers (Figs 6 and 7). These infiltrates were mostly perivascular and consisted of mononuclear cells, some small lymphocytes, some reticulum cells, and, in a few cases, plasma cells. Nodules of lymphocytes, resembling germinal centres, were seen in the lungs of three of the mice at this stage (Fig. 6). The third and final stage was seen in one 6-month-old Balb/c recipient of 25×10^6 F1 spleen cells, and one 5-month-old C57Bl/6 recipient of 50×10^6 F1 spleen cells. In the Balb/c mouse there was almost total replacement of white pulp of spleen and lymph node with reticular cells (Fig. 8). Many of these cells were in mitosis. In the C57Bl/6 mouse reticular cells replaced splenic white pulp, but there was still some recognizable architecture in lymph node, which had germinal centres in the cortex and many pyroninophilic cells, including a few plasma cells, in the medullary cords. The paracortex was however almost replaced by reticulum cells, and there were few small lymphocytes left (Fig. 9). The thymus of this mouse was apparently normal (Fig. 10), but the paracortical lymph nodes were enormously enlarged. Both lung and liver in the stage three mice showed extensive infiltration of mononuclear cells, many of which were reticular cells, some in mitosis, but small lymphocytes were also present, as well as some pyroninophilic cells and frank plasma cells (Fig. 11).

Although it was possible to grade the lesions histologically, there was no apparent correlation of the stages with age. Moderately severe changes were seen in some 2-month-old mice, whilst in mice of 6 months old, all stages were seen. There was no apparent correla-

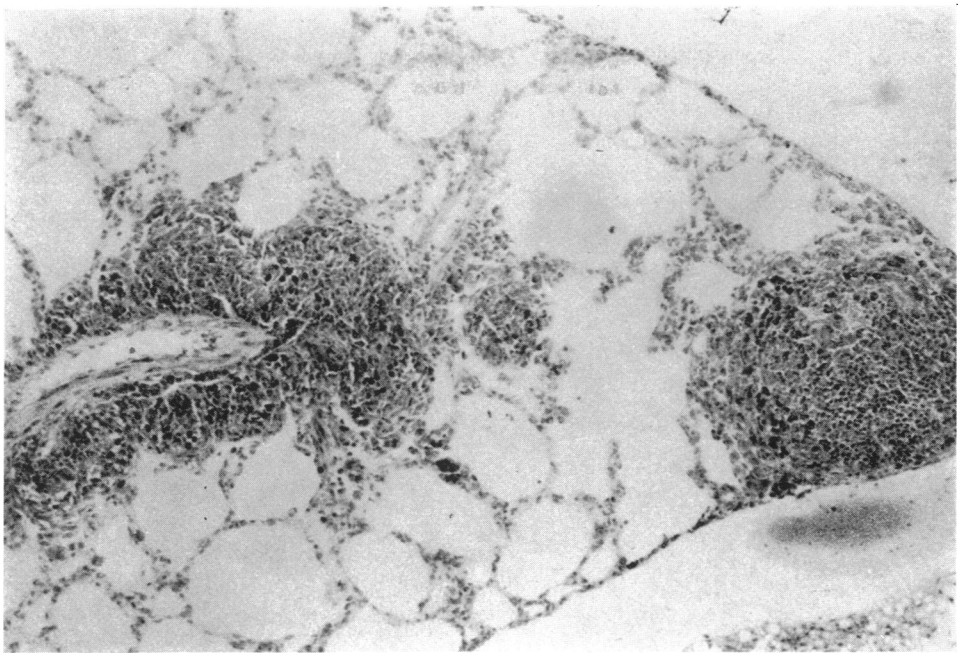

FIG. 6. Lung from a 6-month-old Balb/c recipient of (C57Bl/6 × Balb/c)F1 cells showing stage 2 features. Perivascular infiltrates of mononuclear cells, including plasma cells, blasts, reticulum cells and a few small lymphocytes. Subpleural nodule of lymphoid tissue, resembling a germinal centre. (Methyl Green–Pyronin; magnification × 120.)

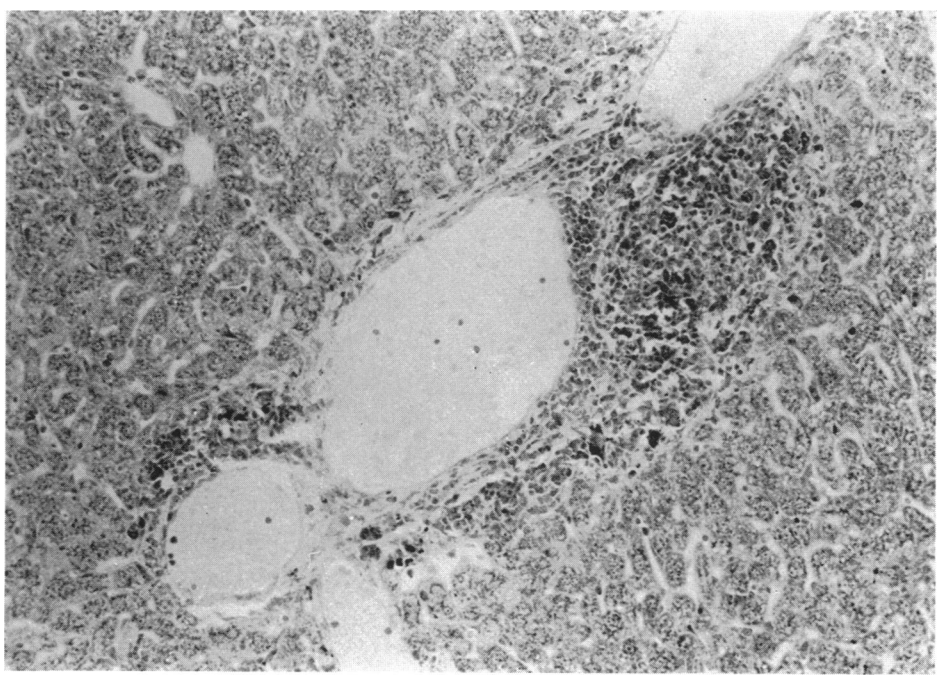

FIG. 7. Liver from same mouse as Fig. 6. Perivascular infiltrates of mononuclear cells. (Methyl Green–Pyronin; magnification × 200.)

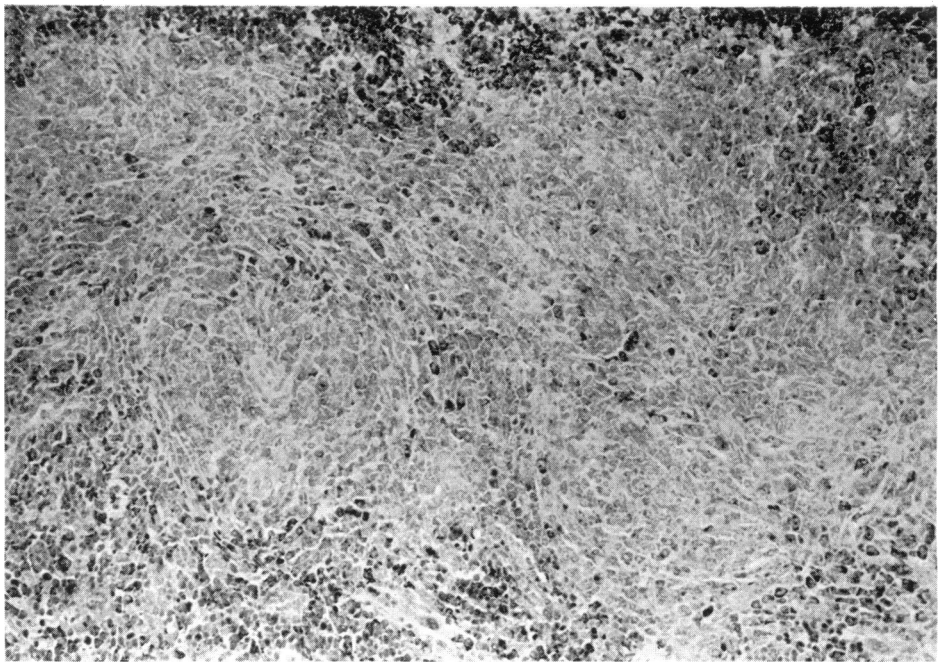

FIG. 8. Spleen from a 5-month-old C57Bl/6 recipient of (C57Bl/6 × Balb/c)F1 cells. Gross pathology shown in Fig. 2. Histologically stage 3: almost total replacement of white pulp by reticular cells. A few pyroninophilic cells are also apparent. (Methyl Green–Pyronin; magnification × 200.)

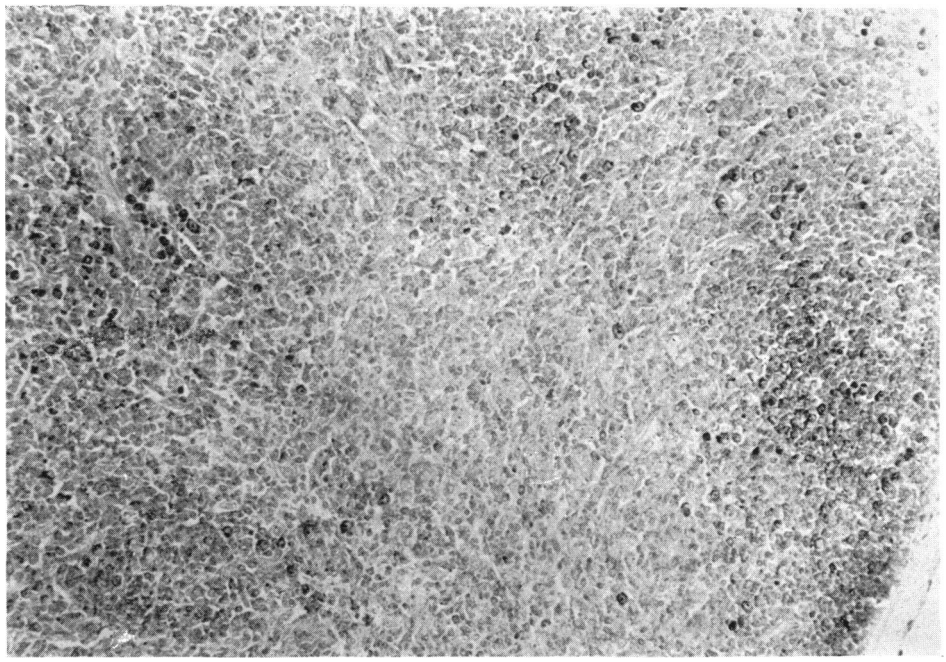

FIG. 9. Lymph node from same mouse as Figs 2 and 8. Stage 3. Germinal centres in cortex, depletion of small lymphocytes in paracortex (thymus-dependent area). (Methyl Green–Pyronin; magnification × 200.)

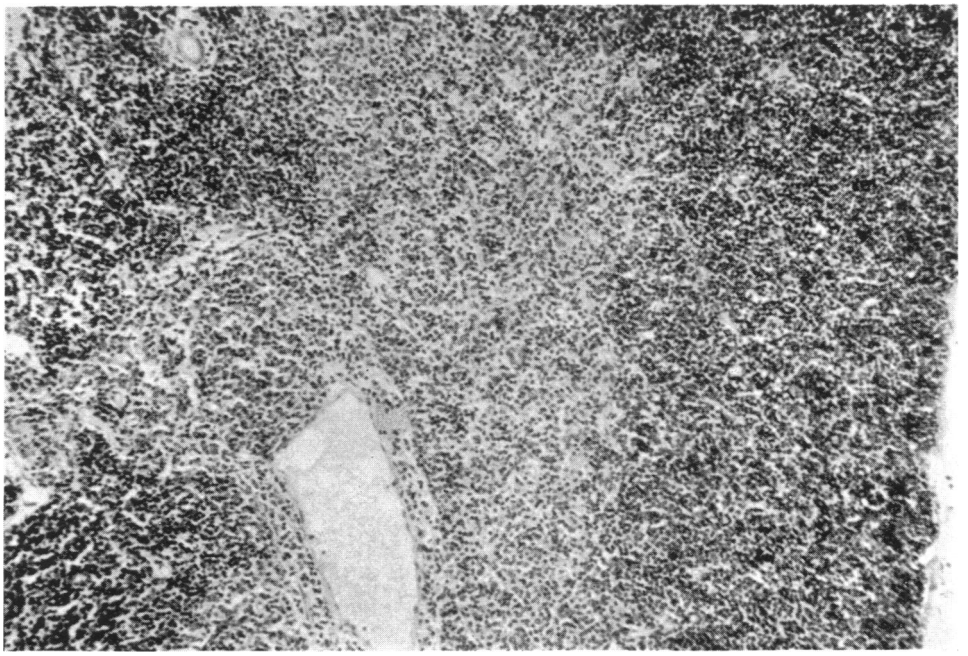

FIG. 10. Thymus from same mouse as Figs 2, 8 and 9. Stage 3. Apparently normal architecture. (Haematoxylin and Eosin; magnification × 148.)

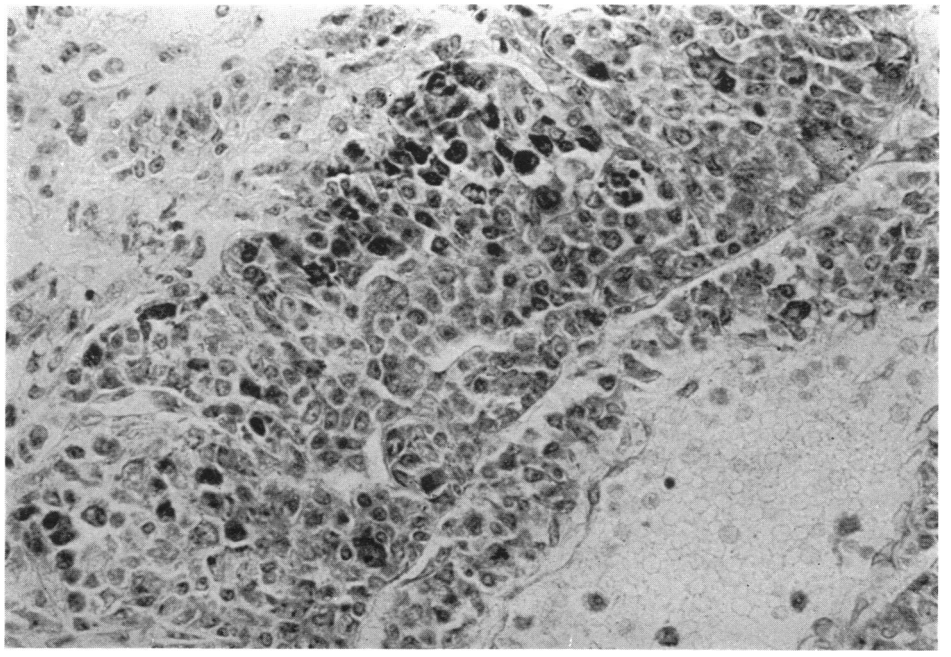

FIG. 11. Lung from same mouse as Figs 2, 8, 9 and 10. Stage 3. Perivascular infiltrate includes reticular cells, pyroninophilic blasts, plasma cells and a few small lymphocytes. (Methyl Green–Pyronin; magnification × 520.)

tion between the number and source of F1 cells given at birth with the severity of the lesions, but as the numbers of animals examined histologically was small, no conclusions can be drawn from this. In no instance did histological examination of a parental recipient of any age, injected neonatally with F1 cells, show a normal picture. All mice had changes, at least in the lymphoid organs. It seems likely that the three stages described form a natural progression of 'the disease' which in many ways looks very much like the chronic pathology of graft-versus-host reaction (Armstrong, Gleichmann, Gleichmann, Beldotti, Andre-Schwartz and Schwartz, 1970).

VIRUS ISOLATION

Table 10 shows that MuL virus was present in two out of three injected animals, but in none of the four control animals. In both cases the virus was detected in Balb/c embryo cells and not in NIH Swiss cells, and is consequently B-cell tropic. None of the animals had developed lymphoma.

TABLE 10

INFECTIOUS MuLV IN SPLEENS OF EXPERIMENTAL ANIMALS AND CONTROLS (NUMBER OF PLAQUE-FORMING UNITS PER 10×10^6 SPLEEN CELLS)

Spleen cells	(C57Bl/6 × Balb/c)F1 cells at birth	Embryo cells	
		NIH Swiss	Balb/c
C57Bl/6 (injected)	50×10^6 (spleen)	0	120
C57Bl/6 (control)	—	0	0
C57Bl/6 (control)	—	0	0
Balb/c (injected)	50×10^6 (bone marrow)	0	10
Balb/c (injected)	50×10^6 (spleen)	0	0
Balb/c (control)	—	0	0
Balb/c (control)	—	0	0

C57Bl/6 mice were 4·5 months old, and Balb/c mice 9·5 months old at the time of assay.

ATTEMPTS TO PASSAGE SPLENOMEGALY

Two F1 spleen-injected Balb/c mice were killed at 6 months of age. Both had splenomegaly, and evidence of abnormality (stages 1 and 2) were confirmed histologically. Spleen cell suspensions were made and groups of (C57Bl/6 × Balb/c)F1 mice, neonatal and young adult, and young adult Balb/c mice were injected intraperitoneally with from $2{\cdot}5 \times 10^6$–$2{\cdot}5 \times 10^7$ cells. At 4 weeks after injection, several mice were killed. No macroscopic abnormality was detected. Three months after injection several more mice were killed, and again, no macroscopic abnormality was seen. The remainder of the mice were left for observation.

DISCUSSION

Runting and splenomegaly are classic signs of allogeneic disease (Simonsen, 1962) but so far we have been unable to discover either the identity of the attacking cell, or the antigen against which the putative attacker is directed.

There would appear to be two possibilities: either the disease is 'graft-versus-host' (GVH), in which case the F1 cells are directing their attack against antigen(s) present on both parental strains, but not on the F1 (compare Ramseier and Lindenmann, 1969), or the disease is 'host-versus-graft' (HVG) and the recipient, instead of developing the expected state of tolerance to F1 antigens, reacts against them and rejects the F1 cells which, initially at least, establish themselves. The second possibility is consistent with some of the observed facts, namely, that according to allotype production, there is evidence for colonization of recipients with functional F1 cells early in life but also for their disappearance later (Table 5). The fact that F1 bone marrow cells (a poor source of T cells) also produce the 'disease' in Balb/c mice indicates that an HVG is more likely than a GVH, since the GVH potential of a cell inoculum is a function of its T-cell content, and bone marrow cells produce poor GVH reactions under condiions where parental cells are injected into F1 hosts (Cantor and Asofsky, 1970).

At variance with the HVG interpretation is the inability to detect cytotoxic effector cells, although perhaps this is not too surprising, as it has proved difficult to show such cells in the usual parent→F1 GVH situation, where even very sensitive assays have picked up only low levels of cytotoxic activity (Cantor, Asofsky, and Wunderlich, unpublished results). It is also possible that GVH effector cells and cytotoxic effector cells are two distinct subpopulations of T cells. A more important objection is the failure to find precursor cells with GVH, MLR or cytotoxic potential when further exposed to alloantigen (Tables 4, 8 and 9). If it is proposed that the recipient parental strain mice become ill and runt during the acute phase of rejecting the F1 graft, supposedly because of its wide and varied tissue distribution, then it would be expected that the potential for reactivity towards these F1 antigens would be retained, if not increased, after rejection (Simpson, O'Hopp and Wunderlich, 1974).

Although the almost total lack of such reactivity is a feature of tolerance induced in parental strain mice of some strains by the injection of F1 cells (e.g. CBA/A→CBA) the concomitant lack of reactivity to third party antigens (compare Beverley *et al.*, 1973) by Balb/c and C57Bl/6 recipients of F1 cells, and their abnormal T-cell function, as s hown by low PHA and Con A responses, indicates a general hyporeactivity rather than classic specific tolerance.

The high background incorporation of [^{3}H]thymidine into unstimulated cultures of these cells indicates that there is a high rate of spontaneous proliferative activity. This is consistent with histological sections of spleen, showing high mitotic activity of an unidentified cell in the thymus-dependent area, enhanced germinal centre (presumably B cell) activity, and diminished numbers of small lymphocytes in the TDA. These findings are accompanied by a marked decrease in the number of theta-bearing cells (unpublished observation) until the final stage of total replacement by reticular cells is reached. It is perhaps not unexpected that they should demonstrate very little T-cell function.

The findings of B tropic leukaemia virus in both C57Bl/6 and Balb/c mice injected with F1 cells are concordant with the observation that when chronic allogeneic disease is induced by injecting adult F1 mice with parental spleen cells, a high incidence of lymphoma is observed in many parent–F1 combinations (Schwartz and Beldotti, 1965; Armstrong *et al.*, 1970; Gleichmann, Gleichmann and Schwartz, 1972). In CAF1 mice injected with Balb/c spleen cells, MuLV was detectable within 1 week after the first injection of parental Balb/c spleen cells (Hirsh, Black, Tracy, Leibowitz and Schwartz, 1970). Virus was also shown to be activated in mixed cultures of Balb/c and CAF1 spleen cells (Hirsh, Phillips,

Solnick, Black, Schwartz and Carpenter, 1972). Moreover, in this combination it has been shown that the *in vivo* activated virus is oncogenic (Armstrong, Black and Richards, 1972; Armstrong, Ruddle, Lipman & Richards, 1973). It is possible that the situation described in this paper is similar, and that during the allogeneic reaction instigated by injecting F1 cells into Balb/c or C57Bl/6 neonates, virus is activated, which then infects and transforms parental lymphoid cells. These cells then become incapable of performing a variety of T-cell functions.

It is not yet clear that the reticulum cell proliferation described here constitutes a true neoplasm, since our initial attempts to transplant have failed. Nonetheless, these findings are best explained by the idea that chronic allogeneic reaction in parental recipients of F1 cells leads to activation of latent endogenous MuLV. The preliminary findings that spleens of two out of three injected animals harboured infectious MuLV, whereas the spleens of non-injected controls did not, appears to support this idea. Definite appraisal, however, of the significance of activated endogenous MuLV in this model must await further experiments with larger groups of animals. The finding that in both C57Bl/6 and Balb/c positive mice the virus was B-cell tropic does not shed light on the question of whether the virus was of parental or F1 origin since both Balb/c and C57Bl/6 carry latent endogenous B-cell tropic viruses (Lilly and Pincus, 1973), which under normal circumstances do not appear in fully infectious form until very late in the life of the animals (Rowe and Hartley, 1972; Stephenson and Aaronson, 1972).

We are also still in the dark as to the nature of the allogeneic reactivity of which runting, splenomegaly and perhaps virus activation, are manifestations. These and the concomitant depression of T-cell function are in sharp contrast to the effects of neonatal injection of some other strains with F1 cells: with the CBA/A combination reactivity does not occur, but specific tolerance is induced. The signals involved in determining reactivity or tolerance might relate to the strength or number of antigenic differences between the pairs, for example, the MLR reaction between Balb/c and C57Bl/6 is greater than that between CBA and A, and this may be a measure of greater antigenic disparity. Alternatively, the number and competence of T cells in the periphery of newborn mice may influence the outcome of injections of F1 cells. If C57Bl/6 and Balb/c mice have larger numbers of competent peripheral T cells at birth than CBA or A mice, they could be more difficult to render tolerant. Both these factors could, separately or together, interact with the presence of endogenous murine leukaemia viruses in determining the outcome.

REFERENCES

Armstrong, M. Y. K., Black, P. H. and Richards, F. F. (1972). 'Tumour induction by cell-free extracts derived from mice with graft-versus-host disease.' *Nature: New Biology*, **235**, 153.

Armstrong, M. Y. K., Gleichmann, E., Gleichmann, H., Beldotti, L., Andre-Schwartz, J. and Schwartz, R. S. (1970). 'Chronic allogeneic disease. II. Development of lymphomas.' *J. exp. Med.*, **132**, 417.

Armstrong, M. Y. K., Ruddle, N. H., Lipman, M. B. and Richards, F. F. (1973). 'Tumour induction by immunologically activated murine leukaemia virus.' *J. exp. Med.*, **137**, 1163.

Beverley, P. C. L., Brent, L., Brooks, C., Medawar, P. B. and Simpson, E. (1973). '*In vitro* reactivity of lymphoid cells from tolerant mice.' *Transplant Proc.*, **5**, 679.

Billingham, R. E., Brent, L. and Medawar, P. B. (1956). 'Quantitative studies on tissue transplantation immunity. III. Actively acquired tolerance.' *Phil. Trans. roy. Soc. (Lond.), B*, **239**, 357.

Billingham, R. E. and Medawar, P. B. (1951). 'The technique of free skin grafting in mammals.' *J. exp. Biol.*, **28**, 385.

Cantor, H. and Asofsky, R. (1970). 'Synergy among lymphoid cells mediating the graft-vs-host (GVH) response. II. Synergy and GVH reactions produced by Balb/c lymphoid cells of differing anatomic origin.' *J. exp. Med.*, **131**, 235.

Canty, T. G. and Wunderlich, J. R. (1970). 'Quantitative *in vitro* assay of cytotoxic cellular immunity.' *J. nat. Cancer Inst.*, **45**, 761.

GLEICHMANN, E., GLEICHMANN, H. and SCHWARTZ, R. S. (1972). 'Immunologic induction of malignant lymphoma: genetic factors in the graft versus host model.' *J. nat. Cancer Inst.*, **49,** 793.

HIRSCH, M. S., BLACK, P. H., TRACY, G. S., LEIBOWITZ, S. and SCHWARTZ, R. S. (1970). 'Leukaemia virus activation in chronic allogeneic disease.' *Proc. nat. Acad. Sci.* (*Wash.*), **67,** 1914.

HIRSCH, M. S., PHILLIPS, S. M., SOLNIK, C., BLACK, P. H., SCHWARTZ, R. S. and CARPENTER, C. B. (1972). 'Activation of leukaemia viruses by graft-versus-host and mixed lymphocyte reactions *in vitro*.' *Proc. nat. Acad. Sci.* (*Wash.*), **69,** 1069.

LANCE, E. M., BOYSE, E. A., COOPER, S. and CARSWELL, E. A. (1971). 'Rejection of skin allografts by irradiation chimeras: Evidence for skin-specific transplantation barrier.' *Transplant. Proc.*, **3,** 864.

LILLY, F. and PINCUS, T. L. (1973). 'Genetic control of murine viral leukaemogenesis.' *Advanc. Cancer Res.*, **17,** 231.

MISHELL, R. I. and DUTTON, R. W. (1967). 'Immunization of associated spleen cell cultures from normal mice.' *J. exp. Med.*, **126,** 423.

MOSIER, D. E. (1973). 'Transient appearance of PHA-reactive thymocytes in the foetal mouse.' *Nature: New Biology*, **242,** 184.

RAMSEIER, H. and LINDENMANN, J. (1969). 'F_1 hybrid animals: reactivity against recognition structures of parental strain cells.' *Path. et Microbiol.* (*Basel*), **34,** 379.

ROWE, W. T. and HARTLEY, J. W. (1972). 'Studies of genetic transmission of murine leukaemia virus by AKR mice. II. Crosses with Fv-1^b strains of mice.' *J. exp. Med.*, **136,** 1286.

ROWE, W. T., PUGH, W. E. and HARTLEY, J. W. (1971). 'Plaque assay techniques for murine leukemia viruses.' *Virology*, **42,** 1136.

SCHWARTZ, R. S. & BELDOTTI, L. (1965). 'Malignant lymphomas following allogeneic disease: transition from an immunological to a neoplastic disorder.' *Science*, **149,** 1511.

SILOBRCIC, V. (1971). 'Life long tolerance and chimerism in parental mice induced with F_1 hybrid cells.' *Europ. J. Immunol.*, **1,** 313.

SIMONSEN, M. (1962). 'Graft-versus-host reactions. Their natural history and applicability as tools of research.' *Progr. Allergy*, **6,** 349.

SIMPSON, E., O'HOPP, S. and WUNDERLICH, J. R. (1974). 'Life span of cytotoxic activity and memory activity following allogeneic skin grafting in the mouse.' *Transplantation.* (In press.)

STEPHENSON, J. R. and AARONSON, L. A. (1972). 'Genetic factors influencing C-type RNA virus induction.' *J. exp. Med.*, **136,** 175.

WUNDERLICH, J. R. and CANTY, T. G. (1970). 'Cell mediated immunity *in vitro*.' *Nature* (*Lond.*), **228,** 62.