

An Investigation of Immunological Tolerance Based on Chimaera Analysis

DONALD MICHIE, M. F. A. WOODRUFF AND IRMGARD M. ZEISS*

Department of Surgical Science, University of Edinburgh

(Received 13th January, 1961)

Summary. Newborn mice of strain A were injected intravenously at birth with 9–15 million spleen cells from adult CBA donors. The recipients exhibited splenomegaly and other signs of graft-versus-host reaction during the first 3 weeks of life. Adult survivors were uniformly tolerant of CBA skin. They showed no sign of a continuing graft-versus-host reaction. The spleens of the treated mice were tested for the presence of immunologically competent donor and host cells by Simonsen's discriminant spleen assay. From the age of 7 days onwards the spleens were found to contain a small percentage of donor cells which were immunologically active against antigens of a third strain. In spleens from adult survivors activity, attributable to the host component, against third-party antigens was undiminished as compared with that of untreated A-strain mice. But activity against the CBA donor strain was absent.

INTRODUCTION

The classical test for the specificity of transplantation tolerance consists in challenging the tolerant animal with grafts of skin and other tissues from the original cell donor (or a member of the same inbred strain) and from a third party. If, as typically happens, the donor-strain graft survives indefinitely but the third-party graft is rejected, the recipient is said to be specifically tolerant of donor-strain tissue, and this condition is attributed by Billingham, Brent and Medawar (1956) to 'an induced specific central failure of the mechanism of immunological response'. This explanation, however, is not the only one possible when, as is usually the case, the injected cells include many which are immunologically competent. In this event the tolerant animal might be a stable lymphoid cell chimaera, harbouring throughout its life a quota of functional cells derived from the original donor, and rejection of third-party grafts might be mediated partly or even exclusively through the activity of those cells. Such rejection would be 'adoptive' by analogy with the way in which a tolerant mouse carrying a skin homograft will reject the graft after being re-equipped with isologous lymphoid tissue (Billingham *et al.*, 1956). The possibility is thus left open that the host component of the 'tolerance chimaera' is immunologically inert rather than specifically tolerant, and that the host is only able to muster an immunological reaction by proxy.

We have sought to dispose of this possibility by the use of Simonsen's discriminant spleen assay (see Simonsen 1960a, 1961), which distinguishes activity of the host component of a chimaera from that of the donor component. This is based on the principle that mice older than 3 days injected with homologous immunologically competent cells

* Present address: Department of Surgery, University of Otago, New Zealand.

develop splenomegaly if, and only if, the injected cells are capable of reacting immunologically against the test animal, and the test animal is genetically disqualified from reacting against the injected cells.

Simonsen's test can be used in a roughly quantitative manner to analyse the spleens of tolerance chimaeras, according to principles which will be explained in more detail in the next section.

The present investigation was designed not only to throw light on the main question outlined above, but also to detect any non-immunological causes which might conceivably contribute to the splenomegaly phenomenon. It is, for instance, within the bounds of possibility that spleen cells transplanted to genetically alien soil might proliferate unduly through disturbance of a balance held by gene-products other than antigens. Into this category might fall the substances responsible for regulating normal tissue growth and regeneration. It is essential to eliminate such a possibility if the splenomegaly test is to gain full reliance as a quantitative and unambiguous immunological assay. The appropriate procedure is to make a spleen assay in which the genetic requirements for the splenomegaly phenomenon are satisfied, but the immunological requirements are not. We accordingly included, in our analysis of tolerance-chimaeras, assays of their spleen cells against newborns of the original donor strain. If the splenomegaly phenomenon is purely immunological, no enlargement should result.

TERMINOLOGY

We shall use the symbol A(CBA) to designate a mouse of strain A tolerant of CBA tissue.

MATERIALS AND METHODS

Tolerance was induced by intravenous injection of adult spleen cells into newborn mice. The spleen donors were from the Harwell substrain of the CBA inbred strain. The recipients belonged to a substrain of the A inbred strain obtained from N. A. Mitchison. The strains used for producing hybrids were C57BL/H from Harwell, DBA-2 from the Chester Beatty Research Institute, and ASW from N. A. Mitchison. At the start of the experiments all the above strains had already been maintained in this department for a number of generations.

THE DESIGN OF THE ASSAYS. Principles for separately assaying the donor and host component of a chimaeric mixture of lymphoid cells have been outlined by Simonsen, *loc. cit.* The crux of the procedure is that the recipient should be genetically qualified to accept one component but to reject the other. To illustrate the principle, we may take from our experimental records an A mouse tolerant of CBA, no. A24, aged 126 days whose spleen was suspected of harbouring lymphoid cells of both A and CBA origin. We wished to detect the presence in the spleen of reactive A cells.

A suspension prepared from the spleen was injected into young hybrid recipients of type A × DBA. What happens to the two types of cell after injection? The A cells are accepted by the host, and at the same time are provoked by the DBA antigens to react against it, with a resultant splenomegaly corresponding in degree to the number of A cells in the inoculum. The CBA cells, on the other hand, are treated as foreign by the A × DBA host and are eliminated before they can cause appreciable splenic enlargement. Clearly the A × DBA host must be old enough to be able to reject the CBA cells. In practice ages

of more than 3 days were found satisfactory. With this proviso, the younger the host the better, since the degree of splenomegaly produced by a given number of cells is inversely related to the age of the host.

The test can be put on a quantitative basis by including injections of normal A spleen cells ('positive controls') at two or more different dose-levels. In order to control the

TABLE 1

SPLEEN WEIGHTS AFTER INJECTION OF SPLEEN CELLS INTO A × DBA HYBRIDS

<i>Source of spleen cells injected into young A × DBA hybrids</i>	<i>No. of cells injected (millions)</i>	<i>Relative spleen weights 10 days after injection (mg./10 g. body weight)</i>	<i>Ratio to negative control</i>
A(CBA)	10	178	2.37
A (positive controls)	11	160	2.13
	5	119	1.59
CBA (negative control)	20	75	—
Uninjected	0	66	—
	0	58	—

possibility that the presence in the chimaeric spleen of donor (CBA) cells might have some spleen-enlarging effect, in spite of the fact that the test hybrids are qualified to eliminate them, other litter-mates must be injected with adult CBA cells. These 'negative controls' are taken as the base-line for assessing effects of treatment.

Table 1 shows the result actually obtained with mouse A24.

The literal inference from this result would be that 10 million A(CBA) cells contained

TABLE 2

SPLEEN WEIGHTS AFTER INJECTION OF SPLEEN CELLS INTO CBA × C57BL HYBRIDS

<i>Source of spleen cells injected into young CBA × C57BL hybrids</i>	<i>No. of cells injected (millions)</i>	<i>Relative spleen weights 10 days after injection. (mg./10 g. body weight)</i>	<i>Ratio to negative controls</i>
A(CBA)	20	46	1.25
	20	54	
CBA (positive controls)	8	109	2.73
	3	56	1.40
A (negative controls)	22	43	—
	22	38	
Uninjected	0	35	—

more than 11 million fully competent host-type cells. This paradoxical conclusion is attributable to sampling and experimental error and can be better expressed by saying that the test shows no impairment of host-type activity in the chimaera. To complete the analysis, a moiety of the same suspension was injected into a second type of hybrid, CBA × C57BL, in order to test for the presence of CBA cells in the suspension. The result in the particular case chosen for illustration is shown in Table 2.

The relative enlargement indicated by the observed ratio of 1.25 shows the presence of donor cells. As a quantitative estimate, we can say that 20 million A(CBA) cells contained more than zero but less than 3 million fully competent donor cells, i.e. that the donor component amounted to less than 15 per cent of the total.

In view of the rather large errors attending a single test of this kind, it is reasonable to ask whether the foregoing example would provide statistically significant evidence of any donor-cell activity at all in the assayed spleen, i.e. whether the 25 per cent excess of the two chimaera-treated mice over the two uninjected controls could be accepted as real. Questions of statistical evaluation are discussed in the following section, in which it will be seen that in our material an excess of 21 per cent of a mean based on two mice over another based on two mice is sufficient for formal significance at the 2.5 per cent level.

NUMERICAL TREATMENT OF THE DATA. Since spleen weight and body weight are highly correlated there is a gain in accuracy if spleen weight is expressed as a ratio, or relative weight, for example as mg. per 10 g. body weight. Comparisons between treated and control animals would also naturally take the form of ratios. Ratios are for several reasons

TABLE 3
ESTIMATES OF VARIABILITY OF RELATIVE SPLEEN WEIGHTS AMONG UN-
TREATED LITTER-MATES IN THE SIX GENOTYPES USED
Analysis of log-transformed data

<i>Age-range</i>	<i>Genotype</i>	<i>Intra-litter sums of squares</i>	<i>Degrees of freedom</i>	<i>Mean square</i>
4-18d	A	0.021,346	12	0.001,696
9-23d	CBA	0.021,647	9	0.002,405
14-18d	A × ASW	0.010,835	6	0.001,806
14-16d	A × C57BL	0.008,166	4	0.002,042
14-20d	A × DBA	0.001,825	2	0.000,912
14-15d	CBA × C57BL	0.009,967	7	0.001,424
All litters combined		0.073,786	40	0.001,845

$(0.001,845)^{\frac{1}{2}} = 0.0430$ = estimated standard deviation on log scale.

awkward to handle. For one thing, there is an algebraic discrepancy between the average of a set of ratios and the ratio of the averages. These difficulties have been avoided by converting all measurements to logarithms before computation, so as to restore the familiar additive relations. After statistical reduction of the log-transformed data, mean ratios and coefficients of variation were obtained by reconversion from a table of anti-logarithms. The means quoted therefore represent geometric and not arithmetic means.

For assessing levels of statistical significance, the coefficient of variation (standard deviation divided by the mean) of relative spleen weights was estimated for normal litter-mates and found to be approximately 10 per cent, corresponding to a standard deviation on the logarithmic scale of 0.0430 which was estimated as shown in Table 3. We may wish to compare the mean of the treated mice in a litter, n_1 in number, with the mean of n_2 control litter-mates. Working in logarithms, we subtract the control from the treated mean. The standard error of the difference is $0.0430 (n_1 + n_2)^{\frac{1}{2}} / (n_1 n_2)^{\frac{1}{2}}$. Hence a difference of more than $1.96 \times 0.0430 (n_1 + n_2)^{\frac{1}{2}} / (n_1 n_2)^{\frac{1}{2}}$ is significant at the 2.5 per cent probability level (not 5 per cent, since we are only interested in one tail of the distribution, i.e. treated > control). The corresponding values after reconversion from logarithms to ratios are set out in Table 4 for various numbers of treated and control mice in a litter.

This tabulation is given as a rough guide: in practice it somewhat overestimates the degree of significance since the intra-litter standard deviation is itself subject to errors of estimation and perhaps also to real fluctuations from one litter to another. It is for this reason that we have taken the more stringent significance level $P < 0.025$ in preference to the conventional $P < 0.05$.

When the evidence from a number of separate litters or tests requires to be combined, weights must be attached to the individual items. Appropriate weights are given by the expression $n_1 n_2 / (n_1 + n_2)$ since the amount of information yielded by an estimate is inversely proportional to the square of its standard error. Standard statistical treatment is then applied, using weighted means and sums of squares instead of the crude unweighted values.

TABLE 4

CRITICAL RATIOS OF TREATED TO CONTROL LITTER-MATES IN RESPECT OF RELATIVE SPLEEN WEIGHTS

If the ratio of the mean of the treated mice to that of the controls equals or exceeds the value shown in the table, a significant effect is indicated at the 2.5 per cent probability level. n_1 = no. of treated mice in litters; n_2 = no. of control mice in litter.

n_1, n_2	Relative spleen weight: ratio of treated to control for significance at 2.5 per cent level.
1,1	1.32
1,2 or 2,1	1.27
1,3 or 3,1	1.25
1,4 or 4,1	1.24
1,5 or 5,1	1.23
1,6 or 6,1	1.23
2,2	1.21
2,3 or 3,2	1.19
2,4 or 4,2	1.18
2,5 or 5,2	1.18
3,3	1.17
3,4 or 4,3	1.16

EXPERIMENTAL PROCEDURES. Cell suspensions were prepared in Hank's solution with the use of a hand-operated glass homogenizer, and passed through a fine stainless steel sieve. The cells were resuspended before use by sucking up and down a few times with a fine Pasteur pipette. Suspensions intended for intravenous injection were washed once, and allowed to stand for 4 minutes before use. These precautions were not found necessary in the case of intraperitoneal injections.

Cell counts were taken over one field of 1 mm.² area at densities ranging from about fifty to about 300 cells per field. When there were any signs of heterogeneity of cell distribution in the counting chamber, four fields were counted and the average was taken.

Skin grafting was carried out by the method of Billingham and Medawar (1951). A graft was classified as successful if it was supple, free from scabbing and growing a full crop of hair at the time that its possessor was used as a spleen donor. Females were

invariably used as skin donors, in order to avoid any complication arising from the Eichwald-Silmser effect (1956).

RESULTS

TOLERANCE AND GRAFT-VERSUS-HOST DISEASE IN MICE INJECTED AT BIRTH WITH HOMOLOGOUS CELLS

Mice of strain A were injected intravenously at birth with numbers of CBA spleen cells ranging from 9 million to 15 million. Survivors were grafted with CBA skin at ages ranging from 5 to 11 weeks. A second group were killed and their spleens weighed and assayed, at intervals during the first 3 weeks of life. These did not receive skin grafts.

The results of skin grafting, summarized in Table 5, show that the doses used were highly effective in inducing tolerance to CBA skin in A-strain recipients. With this strain-combination homografts are regularly rejected within 2 weeks and this was confirmed in seven untreated A mice which were grafted with CBA skin.

The four delayed deaths among the twenty-six injected animals are suggestive of graft-

TABLE 5
INCIDENCE OF TOLERANCE AND RUNT DISEASE IN A-STRAIN MICE, INJECTED AT BIRTH WITH CBA CELLS

Litter	Dose	No. injected	No. died 2nd-4th week	No. grafted	No. grafts accepted	Age of mouse when killed (days)	Age of graft when killed (days)
A24	12×10^6	2	1	1	1	126	47
A16	10×10^6	5	—	5	5	73, 92, 100	21, 70, 78, S, S
A18	14×10^6	1	—	1	1	100	36
A33	10×10^6	4	—	1	1	90	25
Z1	15×10^6	5	—	3	3	82	46, S, S
Z2	15×10^6	5	1	3	3	62, 75, 77	26, 39, 41
Z7	$9-15 \times 10^6$	4	2	2	2	64, 66	17, 19
	Total	26	4	16	16		

S=graft surviving at time of writing.

versus-host disease. No such deaths occurred among eleven uninjected litter-mates. Further evidence is provided by the development of splenomegaly in the younger group of treated animals, which were killed before they were of an age for skin-grafting. The relevant data for this age-group are given in Table 6. The ratios shown may slightly overestimate the effects of treatment, since comparisons were made with uninjected litter-mates rather than with litter-mates receiving similar numbers of isologous cells. However, three additional litters used for other purposes contained litter-mate controls of this type, from which it appeared that the injection of 20 million isologous cells was without effect.

No splenomegaly persisted in the older A(CBA) mice of Table 5. The mean relative weights when killed were 55 and 56 mg. per 10 g. body weight in the treated animals and their untreated litter-mates respectively. This suggestion that the graft-versus-host reaction had spent itself in the older animals was confirmed by determining the phagocytic index in four of the latter. Howard (1961) has shown that the phagocytic index calculated from the rate of removal from the blood stream of intravenously injected colloidal carbon is greatly increased in animals undergoing a graft-versus-host reaction. In all four adult

tolerant A animals which were tested the rates of removal were within normal limits. By contrast, one 15-day-old treated animal of the younger group was tested and gave a phagocytic index of 0.043, as compared with 0.018 for a 14-day-old untreated control. This latter value falls within the normal range, taken to be 0.010–0.020. The relative retardation of growth seen in the younger group of treated animals (Table 6) is doubtless

TABLE 6

BODY WEIGHTS AND MEAN RELATIVE SPLEEN WEIGHT RATIOS OF TREATED AND CONTROL A-STRAIN MICE IN THE YOUNGER AGE GROUP

Asterisks indicate statistical significance according to the criteria of Table 4. n_1 =no. of mice in litter injected with adult CBA cells. n_2 =no. of uninjected litter-mate controls

Litter	Dose in millions of cells	Age at sacrifice (days)	Treated mice		Litter-mate controls		Relative spleen weights: ratio of treated to control
			n_1	wt.(g.)	n_2	wt.(g.)	
Z25	12–15	4	4	2.85	3	2.37	1.40*
Z24	13	4	4	1.86	2	1.69	1.51*
Z23	15	4	1	2.06	2	2.03	1.66*
Z22	15	6	1	2.22	2	3.31	1.67*
Z28	15	7	5	2.56	3	2.65	1.46*
Z21	15	12	2	2.55	1	3.51	2.04*
Z20	14	14	2	5.35	2	6.29	1.81*
Z18	10	14	2	3.14	2	4.68	1.29*
Z16	14	20	1	4.07	1	9.28	0.77

also a reflection of graft-versus-host disease. Two animals in particular showed severe runting, including the single treated member of Z16. In the manner typical of later stages of severe runt disease, this animal had a *small* spleen containing relatively few cells — only 0.64×10^6 per mg. spleen weight in contrast to values ranging from 0.90×10^6 to 2.60×10^6 for the other animals of this group. Otherwise no consistent differences of cellularity appeared between treated and control mice.

ANALYSIS OF THE A(CBA) ADULTS

Assay of the Donor Component

Cell suspensions prepared from the spleens of graft-bearing tolerant mice were injected intraperitoneally into young CBA \times C57BL F₁ hybrids. Positive and negative controls were included in each litter as explained in the section on assay design. After 10 days the test litters of F₁ hybrids were killed, and their body weights and spleen weights recorded. The results are summarized in the left-hand division of Table 7.

Significant evidence of the presence of immunologically competent donor cells was found in five out of nine tested animals. The quantitative sensitivity of the tests was low, so that it was possible only to set approximate limits to the proportion of such cells in the tested cell-populations, but the general conclusion emerges that competent donor cells constituted a minority, perhaps a small one, of the competent cells present.

TABLE 7
 SPLEEN-ASSAYS OF ADULT TOLERANT A MICE BEARING CBA SKIN GRAFTS
 The symbol << implies a positive-control/treated ratio of at least 1.5. Estimates apparently in excess of 100 per cent are attributable to sampling variation

Litter	Age at assay	Tests of donor component against CBA × C ₅₇ BL hybrids		Tests of host component against various hybrids		
		Evidence of presence of competent donor cells	Estimated per cent donor cells	Hybrids used for testing	Evidence of presence of competent host cells	
Z 2	62 days	-	> 27	A × ASW	Highly significant	~ 100
Z 7	64 days	Highly significant	< 9	A × C ₅₇ BL	Highly significant	63
Z 7	66 days	Highly significant	< 18	A × C ₅₇ BL	Highly significant	> 25, < 50
A16	73 days	Not significant	< 20	A × DBA	Highly significant	> 100
Z 2	75 days	Not significant	< 25, > 10	A × ASW	Highly significant	61
Z 2	77 days	Significant	Suitable positive controls lacking	A × ASW	Highly significant	< 100
Z 1	82 days	Not significant	-	A × ASW	Highly significant	> 100
A33	90 days	-	< < 29	A × C ₅₇	Highly significant	> 100
A16	92 days	Not significant	< 16	A × C ₅₇	Highly significant	37
A18	100 days	Highly significant	-	A × ASW	Highly significant	> 36
A24	126 days	Significant	< 15	A × DBA	Highly significant	> 100

The failure to detect a donor component in some of the older animals suggested that the immunological reaction of tolerant animals against third-party antigens was unlikely to be entirely due to the vicarious activity of persisting donor cells. This presumption was confirmed by the results of assaying the *host* component of some of the older tolerant animals, as will now be described.

Assay of the host component. Cell suspensions from the spleens of adult A mice bearing CBA skin grafts were injected intraperitoneally into hybrids between the A strain and one or other of the three strains ASW, DBA-2 and C57BL.

The results of the assays are shown in the right-hand division of Table 7.

An immunologically competent host component was detected in every case. The quantitative evidence gives no ground for supposing that the host component was to any degree impaired in its competence to react to antigens of a third strain. The tolerance of CBA skin exhibited by the assayed animals thus represents a specific inhibition of reactivity. Further evidence for this conclusion accrued from the assays of the spleens of tolerant A mice against newborn CBA mice, as described below.

ASSAY OF SPECIFIC TOLERANCE IN THE HOST COMPONENT

Cell suspensions from the spleens of adult A mice bearing CBA skin grafts were injected intravenously into newborn CBA mice. In the main the suspensions represented moieties of those used in the previous assay.

There are very few antigens of the H-2 system present in CBA which are absent from A (see Gorer, 1959; Gorer and Mikulska, 1959); it was therefore expected that control injections of cells from normal A donors into CBA newborns would give a comparatively poor reaction and one which might take longer to reach its maximum than the 10-day

TABLE 8

RISE AND FALL OF SPLENOmegALY IN CBA MICE WHICH RECEIVED ADULT A-STRAIN SPLEEN CELLS INTRAVENOUSLY AT BIRTH

Figures in bold type denote treated : control ratio of relative spleen weights. Figures in brackets denote numbers of treated and control mice respectively in the litter

Dose	Days					
	9	10	11	12	15	23
5	—	—	—	1.6 (2,3)	—	1.0 (1,2)
10	—	1.9 (2,2)	—	2.7 (1,2)	1.2 (1,2)	1.0 (1,2)
15	—	2.2 (1,2)	—	3.5 (1,3)	—	—
20	1.4 (2,2)	—	2.6 (1,2)	—	—	—
30	—	2.2 (1,2)	—	—	—	—

interval previously used. Pilot tests, summarized in Table 8, confirmed this expectation, and an interval of 12 days was adopted for the assays described in this section.

As implied in the introduction, this assay of the chimaeric A(CBA) spleens is expected to give a null result on the hypothesis that the splenomegaly phenomenon is exclusively immunological in origin. That is to say, no greater spleen enlargement is expected in the animals receiving cells from tolerant A spleens than in negative control litter-mates receiving an equal number of isologous CBA cells. The latter are included in order to

control the possibility that the mere injection of large numbers of spleen cells might cause some spleen enlargement.

Four litters were obtained containing both tolerant-spleen-injected mice and negative controls, permitting the direct comparison to be made as shown in Table 9, in which an additional litter lacking negative controls is included. By the procedures outlined in the section on numerical analysis, a combined estimate was made of the ratio of the mean relative spleen weight of CBA newborns injected with A(CBA) cells to that of litter-mate controls receiving an equal number of CBA cells. The estimate was slightly below unity with an upper fiducial limit of 1.10 at the 2.5 per cent probability level.

There is thus no sign of a spleen-enlarging effect of adult A(CBA) cells when injected

TABLE 9
ASSAY OF SPLEEN CELLS FROM A(CBA) MICE AGAINST NEWBORN CBA RECIPIENTS

<i>A(CBA) donors</i>		<i>Test litters. No. of mice receiving:</i>			<i>Relative spleen weight ratios:</i>	
<i>Litter</i>	<i>Age</i>	<i>(a) 20 × 10⁶ CBA cells (neg. controls)</i>	<i>(b) 20 × 10⁶ A(CBA) cells</i>	<i>(c) 5 × 10⁶ A cells (pos. controls)</i>	<i>(b)/(a)</i>	<i>(c)/(b)</i>
A16	73	—	1	1	—	1.26
A33	90	2	3	2	0.99	1.57
A16	100	1	1	1	0.84	1.25
A18	100	1	3	2	1.04	1.55
A24	126	1	2	—	1.03	—

Combined estimates: $(b)/(a) = 0.984 \pm 0.057$; $(c)/(b) = 1.461$

into CBA newborns at a dosage of 20 million. By contrast the injection of only 5 million cells from non-tolerant A mice ('positive controls') caused substantial enlargement. Three of the four litters referred to above contained satisfactory positive controls and an additional litter of this type was available from which the negative controls were lacking. The spleen enlargement found in these positive controls is entered in the right-hand column of the table, in the form of a ratio to the relative spleen weights of their chimaera-treated litter mates. The combined estimate shows a 46 per cent enlargement.

In sum, the spleen of an adult A mouse tolerant to CBA skin has lost its power to induce splenomegaly in a newborn CBA mouse, although retaining full power against hybrids between A and a third strain.

ASSAY OF DONOR COMPONENT IN THE YOUNGER GROUP

Immunologically competent cells of donor origin can be detected, as we have seen, in the spleen of a tolerant mouse of at least 4 months of age. Is this degree of lymphoid cell chimaerism gradually attained, e.g. by differential multiplication of donor cells, or has it been fully present from the early days of life?

This question was approached by performing on the younger group of animals a series of assays for the donor component similar in all essentials to those done on the adult A (CBA) mice. The results of the assays are shown in Table 10. They are essentially similar to those obtained with the older group, thus giving no evidence of a gradual increase in the degree of chimaerism. The possibility, however, of such an increase having occurred before day 7, the youngest age at which a donor component was detected, is not excluded.

DISCUSSION

The experiments reported above establish rigorously two conclusions about the constitution and status of animals made tolerant of homografts by injecting them at birth with homologous spleen cells.

PRESENCE AND STATUS OF DONOR CELLS

In the first place some of the immunologically competent cells in these animals are of donor type. In other words, the animals are chimaeras, and some at least of the persisting donor cells, or their direct descendants, have retained their immunological competence. This was not unexpected, but hitherto formal proof appears to have been lacking. In the case of animals rendered tolerant by neonatal injection of homologous cells, chimaerism was inferred by Billingham and Brent (1959) from their demonstration that donor-strain antigen persists in these animals. Their evidence, although highly suggestive, is not conclusive, since purified protein antigen can persist for many months in a tolerant reci-

TABLE 10

TESTS OF THE DONOR COMPONENT OF YOUNG A(CBA) MICE AGAINST CBA × C57BL HYBRIDS

In the first six cases the spleens of two or more litter-mates have been pooled as indicated by the numbers after the oblique stroke

<i>Litter and no. of chimaeras</i>	<i>Age in days at assay</i>	<i>Evidence of presence of competent donor cells</i>	<i>Estimated per cent donor cells</i>
Z24/1+3	4	Not significant	< 33
Z24/2+4	4	Not significant	< 33
Z25/1, 3, 4, 5	4	Not significant	< 5
Z28/1-5	7	Significant	11-23
Z21/2+3	12	Not significant	No quantitative estimate
Z20/2+3	14	Highly significant	"
Z18/2	14	Not significant	"
Z26/3	18	Not significant	"
Z16/3	20	Highly significant	"

ipient which is not a chimaera (Dixon and Maurer, 1955). The postulated chimaerism is, however, fully confirmed by the present study, and has been shown to extend to immunologically competent cells, presumably belonging to the lymphoid series.

Chimaerism has on the other hand been sufficiently demonstrated in certain dizygotic twins in cattle and humans, and in lethally irradiated animals resuscitated with homologous haemopoietic tissue (for review see Woodruff, 1960). Some of the evidence, based on the demonstration of donor-type antigens, is perhaps not quite decisive, but morphological evidence, based on female sex chromatin in the human male twin (Booth, Plaut, James, Ikin, Moores, Sanger and Race, 1957), and an easily recognized somatic chromosome marker in the mouse (Ford, Hamerton, Barnes and Loutit, 1956) is unimpeachable.

In the early stages of the chimaerism which we studied, the donor cells were shown to be actively engaged in a reaction against the host. In long-established chimaeras, however, this reaction had subsided, as judged by spleen weights and phagocytic indices. Since they nevertheless retained the competence to react against third-party antigens, it appears that the donor cell population, although initially derived from adult animals, itself became specifically tolerant of the host. The results thus support the claim to this effect

advanced by Simonsen (1960b), and suggest that in our A(CBA) chimaeras a mutual accommodation was ultimately reached between donor and host components, each acquiring specific tolerance of the other.

PRESENCE AND STATUS OF HOST CELLS

Secondly, the great majority of immunologically competent cells in the chimaeras we have investigated are of host type. In other words the notion that rejection of third-party grafts by apparently tolerant animals might be due to cells of donor origin having 'taken over' completely the immunological defences of the animal, can now be rejected, and the original concept of tolerance as immunologically specific is confirmed.

A by-product of the investigation is the demonstration that specific tolerance is a property not only of whole animals but also of aggregations of cells, to wit the spleen-cell suspensions we have employed for the assay of the host component against donor-strain newborns. Whether or not specific tolerance is also a property of individual cells (see Woodruff, 1959) remains open to question, because if one accepts an elective interpretation of immunological specificity (Burnet, 1957; Lederberg, 1959) the phenomenon we have observed can be explained equally well by selective clonal elimination.

ACKNOWLEDGMENT

One of us (I. M. Z.) was granted study leave from the University of Otago in order to take part in this work, and wishes also to acknowledge the hospitality and facilities offered for the purpose by the Department of Surgical Science, University of Edinburgh, in which the work was done.

REFERENCES

- BILLINGHAM, R. E. and BRENT, L. (1959). 'Quantitative studies on tissue transplantation immunity. IV. Induction of tolerance in newborn mice and studies on the phenomenon of runt disease.' *Phil. Trans.*, **242**, 439-77.
- BILLINGHAM, R. E., BRENT, L. and MEDAWAR, P. B. (1956). 'Quantitative studies on tissue transplantation immunity. III. Actively acquired tolerance.' *Phil. Trans.*, **239**, 357-414.
- BILLINGHAM, R. E. and MEDAWAR, P. B. (1951). 'The technique of free skin grafting in mammals.' *Brit. J. exp. Biol.*, **28**, 385-402.
- BOOTH, P. B., PLAUT, G., JAMES, J. D., IKIN, E. W., MOORES, P., SANGER, R. and RACE, R. R. (1957). 'Blood chimerism in a pair of twins.' *Brit. med. J.*, **1**, 1456-8.
- BURNET, F. M. (1957). 'A modification of Jerne's theory of antibody production using the concept of clonal selection.' *Aust. J. Sci.*, **20**, 67-8.
- DIXON, F. J. and MAURER, P. H. (1955). 'Immunologic unresponsiveness induced by protein antigens.' *J. exp. Med.*, **101**, 245-57.
- EICHWALD, E. J. and SILMSER, C. R. (1956). 'The genetics of skin grafting.' *Transplant. Bull.*, **3**, 67.
- FORD, C. E., HAMERTON, J. L., BARNES, D. W. H. and LOUTIT, J. F. (1956). 'Cytological identification of radiation-chimaeras.' *Nature, Lond.*, **177**, 452-4.
- GORER, P. A. (1959). 'Some recent data on the H-2 system of mice.' In: *Biological Problems of Grafting*. Les Congrès et Colloques de l'Université de Liège, **12**, 25-33.
- GORER, P. A. and MIKULSKA, Z. B. (1959). 'Some further data on the H-2 system of antigens.' *Proc. roy. Soc., B*, **151**, 57-69.
- HOWARD, J. G. (1961). 'Changes in the activity of the reticuloendothelial system following the injection of parental spleen cells into F₁ hybrid mice.' *Brit. J. exp. Path.*, **42**, 72-82.
- LEDERBERG, J. (1959). 'Genes and antibodies.' *Science*, **129**, 1649-53.
- SIMONSEN, M. (1960a). 'Identification of immunologically competent cells.' In: *Ciba Symposium on Cellular Aspects of Immunity*. Churchill, London.
- SIMONSEN, M. (1960b). 'On the acquisition of tolerance by adult cells.' *Ann. N.Y. Acad. Sci.*, **87**, 382-7.
- SIMONSEN, M. (1961). 'Graft-versus-host reactions, their history and applicability as tools of research.' *Progress in Allergy*. Basle, Karger.
- WOODRUFF, M. F. A. (1959). Contribution to discussions in: *Biological Problems of Grafting*. Les Congrès et Colloques de l'Université de Liège, **12**, 237 and 258.
- WOODRUFF, M. F. A. (1960). *The Transplantation of Tissues and Organs*. Charles C. Thomas, Springfield, U.S.A.