THE SPECIFIC ANTIGEN-BINDING CELL POPULATIONS OF INDIVIDUAL FETAL MOUSE SPLEENS: REPERTOIRE COMPOSITION, SIZE, AND GENETIC CONTROL*

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During development, an embryonic mouse generates a large number of lymphoid cells, each committed to make antibody molecules of a single specificity. Together, these cells constitute a repertoire capable of recognizing specifically a great variety of foreign antigens. Previous studies have suggested that within the limits of precision of the assays used this repertoire is filled early, with a wide range of specificities, and in the absence of selection by foreign antigens (1, 2).

To determine the extent and nature of individual variation in the development of specific antigen-binding cells, the numbers of cells specific for each of two antigens in the spleens of individual random-bred Swiss-L and inbred CBA/J and BALB/c fetal mice have been measured as a function of spleen size, which was assayed by determining the number of nucleated cells. For Swiss-L fetuses, the ratio of antigen-binding cells to nucleated cells varied more from individual to individual than would be expected from sampling fluctuation. For each inbred strain, however, the number of cells specific for a given antigen was a constant proportion of the total number of nucleated cells within sampling error. These proportions varied from antigen to antigen, and from strain to strain. The ratio of the proportions of cells specific for the two antigens, however, was the same for both CBA/J and BALB/c mice. These results confirmed at the level of the individual fetus the uniform pattern of development seen for populations of fetuses, and suggested that the development of these cells might be subject to strong genetic controls (3).

In order to determine more precisely the genetic and physiological basis of these controls on the developing immune system, we have now measured in these inbred fetuses the numbers of spleen cells specific for each of four antigens as a function both of the number of nucleated cells per spleen and of the number of immunoglobulin (Ig)-bearing cells per spleen. In addition, we have determined the numbers of antigen-specific cells as a function of spleen cell number in a variety of F_1 and backcross hybrid fetuses. Our data suggest that there are at least two independent groups of controls affecting the development of antigen-binding cells, both of which are distinguishable from controls on the overall

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development of the mouse. The first appears to determine the composition of the repertoire, i.e., the relative numbers of cells specific for each antigen. The second determines the rate at which this repertoire is expressed in the course of development, and appears to be subject largely to the action of one or a few simple Mendelian genes.

Materials and Methods

Fetal Mice. To obtain fetuses of known gestational age, three to five female mice were caged together with one male and inspected daily for vaginal plugs. The day a plug was found was taken to be day 0 of gestation. Breeding colonies were maintained of random-bred Swiss-L mice (1), and of various combinations of inbred BALB/c (*H-2*^{d}), CHA/J (*H-2*^{k}), and F_1 mice. On the 18th day of gestation, every fetus in a litter was removed from the mother, counted, and weighed to the nearest 0.01 g.

Antigens. Sheep erythrocytes (SRBC; Microbiological Associates, Bethesda, Md.; obtained from sheep nos. 5, 7, 8, 18, 25, 26, or 35) were stored at 4°C and used within 10 days of removal from the sheep. SRBC were derivatized with the 2,4,6-trinitrophenyl (TNP)[1] hapten by the method of Rittenberg and Pratt (4). To derivatize SRBC with the *p*-toluenesulfonyl (Tosyl) hapten, one volume of washed, packed SRBC was suspended in seven volumes of a solution of 0.7 mg/ml *N*-tosyl-ε-amino-caproic acid (5) and 1.4 mg/ml 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulfonate (Aldrich Chemical Co., Inc., Milwaukee, Wis.) in 0.28 M sodium cacodylate, pH 6.9, that had been preincubated for 30 min at room temperature and centrifuged to remove any sediment. The cell suspension was incubated 15 min at room temperature in this solution, then washed twice in modified barbital buffer (MBB) (4) plus 0.55 mg/ml glycyl glycine, and three times in plain MBB. In all cases, the SRBC were washed with MBB and spun at low speed to remove any erythrocyte clumps immediately before use.

Detection of Antigen-Binding and Ig-Bearing (Ig+) Cells. Cell suspensions were prepared from the spleens of individual 18-day fetal mice, and assayed for cells capable of binding SRBC or antigen-coated SRBC to form rosettes as described previously (2, 3). To determine the number of cells specific for each of two antigens in a single spleen, the cell suspension was divided into two equal portions, each of which was assayed for rosette-forming cells (RFC) by the usual procedure, but using half the usual amounts of all reagents.

To determine the fraction of Ig+ cells in single fetal spleens, the cells obtained from one spleen were suspended in 0.15 ml of Eagle's minimum essential medium supplemented with 1% heat-inactivated fetal calf serum (both from Microbiological Associates) and 10 mM NaN_3 (MFA buffer). Fluorescein-conjugated rabbit antibodies against mouse Ig were added to the cell suspension to give a final antibody concentration of 0.5 mg/ml, the mixture was incubated 15 min at 37°C, washed once with MFA buffer, resuspended in 0.1 ml MFA buffer, and examined microscopically to determine the fraction of viable nucleated cells that was labeled with the fluorescein-conjugated antibodies (1).

Statistical Methods. To determine whether the proportion of RFC to nucleated cells varied among any number k of fetuses, the counts of RFC were arranged in the first row of a $2 \times k$ contingency table, the corresponding counts of nucleated cells were arranged in the second row, and the observed proportions were tested for goodness of fit to the proportion which would be expected if the proportion were the same for all the fetuses in the table, i.e., whether, on a plot of RFC number as a function of nucleated cell number, the data approximate a straight line through the origin (6, p. 232). A new statistical procedure for testing whether a straight line not necessarily through the origin adequately describes such cell counts has been derived (J. E. Cohen, P. D'Eustachio, and G. M. Edelman, unpublished observations).[2] In either case, goodness of fit was

[1] *Abbreviations used in this paper: h*, dominance; Ig+, immunoglobulin bearing; MBB, modified barbital buffer; MFA buffer, Eagle's minimum essential medium supplemented with fetal calf serum and NaN_3; *n*, number of loci controlling a difference between two inbred strains, RFC, rosette-forming cells; TNP, trinitrophenyl; Tosyl, *p*-toluenesulfonyl; Var. variance.

[2] A preliminary description of this procedure has been deposited with the National Auxiliary Publications Service, c/o Microfiche Publications, P. O. Box 3513, New York 10017 (NAPS Accession no. 03035).

measured by the G^2 statistic (7). These techniques are not equivalent to conventional linear regression, which is inappropriate for these data. To determine whether the ratio of the proportion of one specific cell population to the proportion of another specific cell population differed from one strain to another, the method of Fleiss (8) was used. All of these statistical tests were performed using the numbers of cells actually observed, rather than any estimates (e.g., total RFC per spleen) derived from these numbers.

Wherever variation is asserted to be within sampling fluctuation, the probability of variation at least as large as that observed due to chance alone is 1% or greater. Wherever a difference is asserted to be statistically significant, the probability of any variation that is at least as large as that observed due to chance alone is less than 1%.

The number of loci (n) controlling the difference between the inbred parental strains CBA/J and BALB/c in the number of TNP-SRBC RFC per 10^5 nucleated cells was estimated using the method of Wright (9). Specifically, preliminary tests showed no significant difference between the reciprocal F_1's in either the mean or the variance (Var) of TNP-SRBC RFC per 10^5 nucleated cells. Therefore the data from BALB/c × CBA/J and from CBA/J × BALB/c crosses were pooled to obtain the mean and variance for the F_1 generation. Dominance (h) of the CBA/J genotype was estimated by h = [Mean(F_1) − Mean(BALB)]/[Mean(CBA) − Mean(BALB)]. Using the parental, F_1, and F_2 genotypes, n was estimated by:

$$n = \frac{[\text{Mean(CBA)} - \text{Mean(BALB)}]^2[1.5\text{-}2\ h(1-h)]}{8(\text{Var}(F_2) - [\text{Var(BALB)} + \text{Var(CBA)} + 2\ \text{Var}(F_1)]/4)}. \quad (1)$$

This estimate of the value of n is relatively insensitive to variation in the value of h. Nevertheless, to avoid assumptions about the uniformity of h at different loci, n was also estimated from parental, F_2, and backcross data by:

$$n = \frac{[\text{Mean(CBA)} - \text{Mean(BALB)}]^2}{8(2\ \text{Var}(F_2) - [\text{Var(BBC)} + \text{Var(CBC)}])}. \quad (2)$$

where BBC = BALB/c × (BALB/c × CBA/J)F_1 and CBC = CBA/J × (BALB/c × CBA/J)F_1. In a modification of these formulas, each variance contributing to the estimates of environmental variance was weighted in proportion to its sample size. With the present data, the numerical results differed negligibly from those given by equations (1) and (2). The analysis was repeated using, for each individual fetus, the square-root of the number of TNP-SRBC RFC per 10^5 nucleated cells, again with negligibly different results. Although no formal estimates appear to be available (9) for the uncertainty to be attached to the estimates of n obtained from equations (1) and (2), the estimates contain the difference of large quantities in the denominators and are likely to be highly variable. Moreover, the theory underlying equations (1) and (2) assumes that because each of the parental and the F_1 stocks should be equally genetically uniform, the variances of the number of TNP-SRBC RFC per 10^5 nucleated cells should be equal in the three stocks. This assumption is contradicted by our observation, using Bartlett's test (6, p. 296), that there were significant differences among the three variances, the F_1 fetuses having the largest variance. The results obtained from equations (1) and (2) therefore indicate no more than the nearest order of magnitude.

Results

Quantitation of Antigen-Binding and Ig+ Cells in the Spleens of Inbred Fetal Mice. The spleens of inbred fetal mice show no significant individual variation in the proportion of TNP-specific cells to nucleated cells, and in the proportion of SRBC-specific cells to nucleated cells (3). In this study, the numerical relationship among the various antigen-specific spleen cell populations within and between strains was determined more precisely using a total of four antigens, and related to the frequency of Ig+ cells in these spleens (Tables I and II).

The proportion of Ig+ spleen cells among nucleated spleen cells, detected by immunofluorescence assays, was essentially constant for all individual fetuses

TABLE I

Numbers of Ig+ and Nucleated Cells in the Spleens of Individual Inbred Mice

Litter serial number	Fetus serial number	Fetal weight	Observed counts		Calculated % Ig+
			Ig+	NC*	
		g			
Mouse strain: CBA/J fetuses					
1	1‡	0.67	6	75	8
	2‡	0.81	4	75	5
2	1‡	1.09	10	150	6
	2‡	0.74	9	100	9
Mouse strain: BALB/c fetuses					
3	1‡	1.13	2	150	1
4	1‡	1.04	1	100	1
5	1‡	1.15	3	162	1
	2‡	1.03	3	150	2
6	1‡	0.98	2	93	2
	2‡	1.03	2	54	3
7	1‡	1.18	2	55	3
	2‡	1.13	0	59	0
Mouse strain: CBA/J adult female					
	1	–	21	55	38
Mouse strain: BALB/c adult females					
	1	–	12	36	33
	2	–	9	47	19
	3	–	13	28	46

* NC, nucleated cells.

‡ All fetuses, but none of the adults, in this table were counted blind.

within a strain, but varied significantly between strains (Table I). The numbers of cells in the spleens of 18-day CBA/J and BALB/c fetuses specific for each of four antigens were counted by means of the rosette assay, and correlated with the numbers of nucleated cells in these spleens (Table II).[3] For each antigen and each strain of mouse, the proportion of RFC among nucleated cells varied no more from individual to individual than would be expected due to sampling fluctuation alone. For each antigen except sheep 26 RBC, however, these proportions varied significantly from strain to strain, and for each strain, the proportions varied significantly among antigens.

The ratio of the RFC frequency observed for CBA/J fetuses to that for BALB/c fetuses was constant, within sampling fluctuation for each antigen, however (Table II A). This same ratio, approximately four, was observed for the frequencies of Ig+ cells. Thus, within the population of Ig+ cells, the relative numbers of cells specific for each of the antigens tested appeared to be constant for both strains of mice, and the difference in the proportions of specific antigen-binding cells between CBA/J fetuses and BALB/c fetuses could be entirely accounted for by the difference between the inbred strains in the proportions of Ig+ cells among nucleated cells. In addition, although the two strains differed in numbers of nucleated cells per spleen and in the proportion of nucleated cells which were

[3] The data for these individual fetuses (weight, RFC number, and nucleated cell number) have been deposited with the National Auxiliary Publications Service (NAPS Accession no. 03035).

TABLE II

Frequencies and Ratios of RFC and Ig+ Cells in the Spleens of Inbred 18-Day Fetal Mice

Mouse strain	TNP-SRBC RFC	Sheep 7 RBC RFC	Sheep 26 RBC RFC	Tosyl RFC	Ig+
(A) Positive cells per 10^5 nucleated cells (mean ± SD)*					
CBA/J	32 ± 12	8 ± 7	12 ± 13	7 ± 5	(7 ± 2) × 10^3
BALB/c	8 ± 2	2 ± 0.4	6 ± 0.2	1 ±0.3	(2 ± 1) × 10^3
Ratio‡	3.8	4.0	1.6	7.0	4.0
(B) Positive cells per spleen§					
CBA/J	70 ± 54	12 ± 8	26 ± 22	12 ± 7	(1.8 ± 2.3‖) × 10^4
BALB/c	58 ± 37	20 ± 5	28 ± 3	6 ± 4	(1.2 ± 1.3‖) × 10^4

* Calculated from the estimated proportions, weighing each fetus equally.

‡ Based on pooled cell counts for each antigen and strain, CBA/J:BALB/c.

§ Calculated from the estimated proportions, except for Ig+ cells, which were calculated on the bases of (2.6 ± 2.6) × 10^5 nucleated cells per spleen for 41 CBA/J fetuses and (7.3 ± 3.2) × 10^5 nucleated cells per spleen for 24 BALB/c fetuses.

‖ Propagated standard deviation.

Ig+, the total numbers of Ig+ cells and antigen-specific cells per spleen were not demonstrably different between strains (Table II B).

In these two inbred strains of mice, the frequency of splenic T cells was low on the 18th day of gestation. No small lymphocytes bearing surface θ-antigen detectable by indirect immunofluorescence (1) were found in the spleens of CBA/J mice. In BALB/c mice, the frequency of such θ-bearing cells was less than half that of Ig+ cells.

Effects of Body Weight and of Littermates. Both the number and the frequency of splenic antigen-specific cells increase during development (1, 2). Because the BALB/c fetuses were generally larger than the CBA/J fetuses (1.055 ± 0.166 g vs. 0.879 ± 0.143 g [mean ± standard deviation]), it is necessary to consider whether the systematic difference between the CBA/J and BALB/c fetuses in the proportion of nucleated spleen cells which are Ig+ might be due to differences in the overall development of the two strains. This appears not to be the case. Although the difference in weight between the 10 heaviest and the 10 lightest CBA/J fetuses assayed for TNP-SRBC RFC was significant, the relative frequencies of TNP-specific spleen cells in the two groups were indistinguishable. Conversely, although the 10 heaviest CBA/J fetuses did not differ significantly in weight from the 10 lightest BALB/c fetuses, the relative frequencies of TNP-specific cells observed in the two groups were significantly different. Similarly, no differences could be found within either inbred strain among fetuses grouped on the basis of litter size or rank in litter, while groups of BALB/c and CBA/J fetuses matched for these parameters did show significant differences.

Frequencies of Antigen-Binding Cells in Random-Bred Fetal Mice. As reported previously (3) the proportions of TNP-SRBC RFC to nucleated cells and of SRBC RFC to nucleated cells vary significantly from one Swiss-L fetal mouse to the next. To study the numerical relationship between TNP-specific cells and SRBC-specific cells, it was therefore necessary to determine the frequencies of both cell types for individual fetal spleens (Table III).

TABLE III

Numbers of RFC for Two Different Antigens and Nucleated Cells in the Spleens of Individual Swiss-L 18-Day Fetal Mice

Litter serial number	Fetus serial number	Fetal weight	Observed sample counts				Calculated estimates			TNP RFC per sheep 26 RFC
			TNP-SRBC RFC*	NC	Sheep 26 RBC RFC	NC	TNP RFC per spleen	Sheep 26 RFC per spleen	10^5 NC per spleen	
		g								
1	1	1.76	153	412	8	318	765	40	9.1	19.1
	2	1.85	91	371	7	361	455	35	9.2	13.0
2	1	1.65	108	321	7	284	540	35	7.6	15.4
	2	1.62	58	316	5	418	290	25	9.2	11.6
Mean		1.72					513	34	8.8	14.8‡
Standard deviation		0.11					198	6	0.8	3.3

* $G^2 = 21.8$ with three df for homogeneity of TNP-SRBC RFC per nucleated cell (NC); $P < 10^{-3}$. Sheep 26 RBC RFC per NC $G^2 = 2.21$ with three df, $P = 0.53$. All experiments in this table were done blind with the equivalent of four aliquots for each antigen assay, using sheep 26 RBC as TNP carrier and as antigen. One fetus tested using sheep 18 RBC is omitted from this table.

‡ The probability that the difference between this ratio and the ratio observed for the same two antigens in BALB/c and CBA/J fetuses is due to sampling fluctuation is less than 10^{-8}.

In Swiss-L fetuses, one half of the cell suspension prepared from an individual spleen was assayed for TNP-SRBC RFC and nucleated cells, while the other half was assayed for SRBC RFC and nucleated cells using RBC from sheep 26. The average number of TNP-SRBC RFC per spleen in these fetuses greatly exceeded that in either inbred strain (Table II), and the variation among individual Swiss-L mice in the proportion of TNP-SRBC RFC among nucleated cells significantly exceeded sampling fluctuation. The variation from individual to individual in the ratio ([TNP-SRBC RFC/nucleated cells]/[sheep 26 SRBC-RFC/nucleated cells]), however, was no greater than would be expected due to sampling fluctuation alone. This ratio of proportions did differ significantly, however, from the ratio for the same two antigens observed for BALB/c and CBA/J fetuses (Table II).

These data suggest the existence of two groups of controls in the developing immune system of the mouse. One regulates the total size of the repertoire, measured as the proportion of antigen-binding or Ig+ cells among nucleated cells; the other affects the composition of this repertoire, that is, the relative numbers of cells specific for each of a panel of test antigens.

Genetic Control of Antigen-Binding Cell Populations. To test the possibility of a genetic basis for the control affecting repertoire size, both TNP-SRBC RFC and nucleated cells were counted in the spleens of F_1, backcross, and F_2 fetuses.

F_1 Hybrid Fetuses. Fetuses from seven BALB/c mothers bred with CBA/J fathers [(BALB/c × CBA/J)F_1 fetuses] were assayed for TNP-SRBC RFC and nucleated cells at 18 days gestation (Table IV). In four of the five litters from which more than one fetus was assayed, the variation among individual fetuses in the proportion of RFC to nucleated cells was within sampling fluctuation. In litter no. 1, the counts of RFC and nucleated cells were well described by assuming that the number of RFC is proportional to the excess of the number of nucleated cells over some threshold (Fig. 1). Graphically, this assumption implies that a scattergram of RFC per spleen along the ordinate, against nucleated cells per spleen along the abscissa, should approximate a straight line

TABLE IV

Numbers of TNP-SRBC RFC and Nucleated Cells in the Spleens of Individual F_1 18-Day Fetal Mice

Litter serial number	Fetus serial number	Fetal weight	Observed sample counts			G^2*	P	Calculated estimates		
			Aliquots	TNP-SRBC RFC	NC			RFC per spleen	10^4 NC per spleen	RFC per 10^5 NC
		g								
Mouse strain: BALB/c × CBA/J‡										
1 (8)§	1	1.45	3	52	337	19.97	10^{-3}‖	347	112	31
	2	1.22	3	6	141			40	47	9
	3	1.24	3	14	177			93	59	16
	4	1.15	3	5	116			33	39	9
	5	0.96	3	5	88			33	29	11
2 (26)	1	1.27	3	23	159	5.91	0.12	153	53	29
	2	1.21	3	40	167			267	56	48
	3	1.41	3	67	299			447	100	45
	4	1.25	3	20	138			133	46	29
3 (8)	1	1.19	6	22	554	1.40	0.50	73	92	8
	2	1.17	2	4	84			40	42	10
	3	1.21	6	21	365			70	61	12
4 (35)	1	1.10	2	3	78	NA		30	39	8
5 (35)	1	1.47	2	36	290	NA		360	145	25
6 (35)	1	1.11	3	11	180	2.96	0.40	73	60	12
	2	1.07	3	4	116			27	39	7
	3	1.09	3	5	120			33	40	8
	4	1.15	3	12	148			80	49	16
7 (26)	1	1.29	3	38	214	4.72	0.19	253	71	36
	2	1.28	3	51	211			340	70	48
	3	1.38	3	104	385			693	128	54
	4	1.30	3	59	277			393	92	43
Mean		1.226						182.4	66.8	23.2
Standard deviation		0.128						180.4	31.7	16.0
Mouse strain: CBA/J × BALB/c¶										
1 (26)	1	1.23	3	25	159	NA		167	53	31
2 (26)	1**	0.87	6	19	176	NA		63	29	22
3 (8)	1	1.10	6	6	200	NA		20	33	6
4 (8)	1	1.19	6	11	280	0.04	0.84	37	47	8
	2	1.11	6	9	250			30	42	7
5 (18)	1	1.17	4	3	192	NA		15	48	3
6 (35)	1	1.07	4	3	198	0.63	0.43	15	50	3
	2	1.01	4	4	144			20	36	6
7 (35)	1	1.06	4	3	166	NA		15	42	4
8 (25)	1	1.01	4	14	100	0.20	0.65	70	25	28
	2	1.10	4	17	144			85	36	24
Mean		1.084						48.8	40.0	12.8
Standard deviation		0.099						46.3	8.9	11.0

* Test of homogeneity within each litter. G^2 should have the distribution of a χ^2 variate with degrees of freedom one less than the number of fetuses tested within the litter. NA, not applicable.

‡ Overall homogeneity $G^2 = 220.6$ with 21 df, $P < 10^{-10}$.

§ The number in parentheses indicates the sheep whose erythrocytes were used in the rosette assays.

‖ Fitting an affine linear model gave an estimate of $d = 26.59$ nucleated cells per aliquot, with $G^2 = 5.21$ and three df, $P = 0.16$.

¶ Overall homogeneity $G^2 = 69.1$ with 10 df, $P < 10^{-10}$.

** Not made blind: all other cell counts in this table were made blind.

which intersects the horizontal axis at the threshold number of nucleated cells per spleen. When the five litters were considered as a single group, however, statistically significant fluctuation was observed, whether the data were tested for fit to the conventional homogeneity model, or for fit to the affine model.

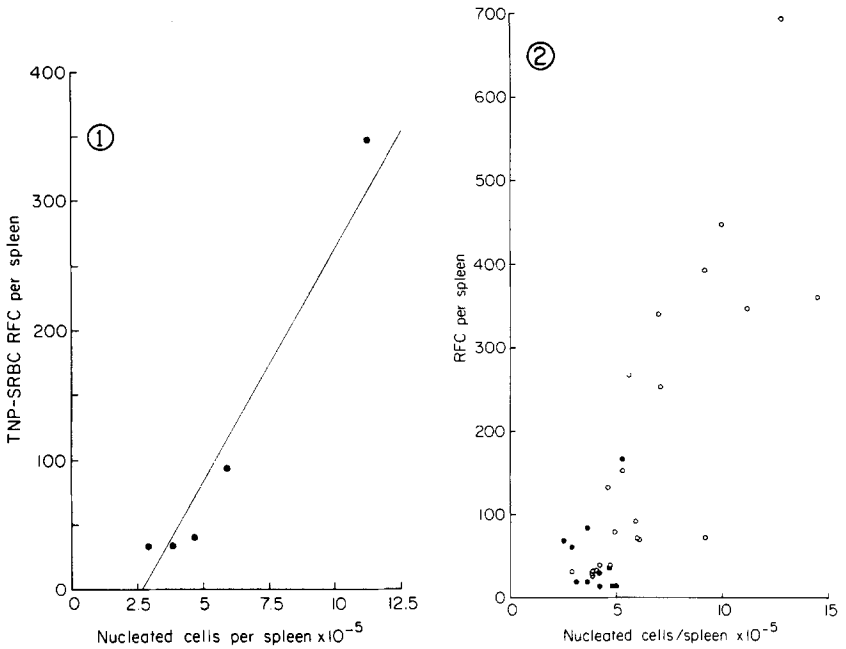

FIG. 1. Numbers of TNP-specific RFC in the spleens of BALB/c × CBA/J fetal mice (litter serial no. 1, Table IV) as a function of size. RFC number per spleen was calculated from the number of RFC observed in 25-μl aliquots of the rosette assay mixture (equivalent to 1/20 spleen). The number of nucleated cells in the spleen was used as an index of its size. Each point (●) represents the results obtained for one spleen. All fetuses were tested on the 18th day of gestation.

FIG. 2. Numbers of TNP-specific RFC in the spleens of all assayed BALB/c × CBA/J (○) and CBA/J × BALB/c (●) F_1 fetal mice (Table IV) in relation to spleen size, as in Fig. 1. Each point represents the results obtained for one spleen. All fetuses were tested on the 18th day of gestation.

Fetuses from eight CBA/J mothers bred with BALB/c fathers [(CBA/J × BALB/c) F_1 fetuses] were assayed for TNP-SRBC RFC and nucleated cells (Table IV). As before, the results for each litter separately were consistent with a linear relationship between the number of RFC and the number of nucleated cells, but when all fetuses were considered together, significant fluctuation was observed.

A scattergram of TNP-SRBC RFC against nucleated cells for all (BALB/c × CBA/J)F_1 and (CBA/J × BALB/c)F_1 individuals (Fig. 2) suggests that in F_1 fetuses the number of RFC per spleen is proportional to the excess of the number of nucleated cells over a threshold of 2.5×10^5. Both crosses adhere to this pattern only roughly, as there is more variation about a straight line than could be explained by sampling fluctuation alone. The constant of proportionality more closely resembles that for inbred CBA/J individuals than that for inbred BALB/c fetuses, however. This result is consistent with the control of RFC relative frequency by a single autosomal Mendelian locus, with the allele at that locus expressed by CBA/J mice dominant to the allele expressed by BALB/c mice.

Backcross and F_2 Fetuses. To test this possibility further, fetuses obtained

from 14 backcrosses between (BALB/c × CBA/J)F_1 males and BALB/c ("recessive" parental) females were assayed for TNP-SRBC RFC and nucleated cells at 18 days gestation (Table V, Fig. 3). This population of fetuses differed from fetuses of each inbred line, and from F_1 fetuses. The population appeared to fall into two distinct groups of approximately equal size, one with a relatively high proportion of RFC to nucleated cells, and the other with a low proportion.

More detailed statistical analysis of within- and between-litter variation was carried out, and compared with the results of parallel analyses carried out for the F_1 fetuses. In 8 of the 10 litters where more than one individual was assayed, the variation among individuals in the proportion of TNP-SRBC RFC was within sampling fluctuation; in the other two of these 10 litters, it was not possible to describe the cell counts adequately by any straight line.

Notwithstanding the apparent homogeneity in the proportion of TNP-SRBC RFC within each of eight litters, these same eight litters taken together provide definite evidence of nonsampling fluctuation in the proportion of RFC that is consistent with genetic segregation. Since each litter is independent, the G^2 statistic testing homogeneity within litters in the proportion of RFC is independent from one litter to another. Hence, the sum of the eight G^2 statistics over the litters which appear to be homogeneous should be distributed as a χ^2 variate with degrees of freedom equal to the sum of the degrees of freedom of each of the eight litters (7). For these eight litters, the sum of the eight G^2 statistics is 36.990 and the sum of the df is 18. Thus, the chance that the within-litter variability in the proportion of RFC would arise by fluctuation alone is less than 0.01.

For comparison, the sum of the three G^2 statistics testing homogeneity in the three litters of (CBA/J × BALB/c)F_1 fetuses with more than one assayed fetus was 0.869 with three df. The sum of the four unrounded G^2 statistics testing homogeneity in the four homogeneous litters of (BALB/c × CBA/J)F_1 fetuses plus the one G^2 statistic testing linearity in litter no. 1 was 19.874, with a corresponding sum of 14 df. Hence, in the F_1 fetuses, the total G^2 statistic testing within-litter linearity over all litters for which the test was accepted was 20.743 with 17 df. Here there is no significant heterogeneity within litters.

A single litter of five CBA/J × (BALB/c × CBA/J) backcross fetuses was assayed for the proportion of TNP-SRBC RFC (Table VI). The proportions varied no more among individuals than expected from sampling fluctuation. The proportions did not differ significantly from those of CBA/J inbred fetuses, consistent with the dominance of some genetic factor(s) controlling the proportion of TNP-SRBC RFC in the inbred CBA/J strain.

Fetuses from two litters of (BALB/c × CBA/J) × (BALB/c × CBA/J)F_2 hybrids were also assayed for the proportion of TNP-SRBC RFC (Table VII). The proportion varied significantly among the five fetuses.

Estimation of the Loci Controlling Repertoire Size. The summary statistics of the preceding breeding experiments were used according to the method of Wright (9) to estimate the number of loci, n, controlling the difference between the CBA/J and BALB/c strains in the proportion of TNP-SRBC RFC to nucleated cells (see Materials and Methods). The mean and standard deviation of the number of TNP-SRBC RFC per 10^5 nucleated cells for the pooled F_1 generation were 19.8 and 15.2, respectively. The estimated dominance, h, was 0.50. The esti-

TABLE V

Numbers of TNP-SRBC RFC and Nucleated Cells in the Spleens of Individual BALB/c × (BALB/c × CBA/J) Backcross 18-Day Fetal Mice

Litter serial number	Fetus serial number	Fetal weight	Observed sample counts			G^{2*}	P	Calculated estimates		
			Aliquots	TNP-SRBC RFC	NC			RFC per spleen	10^4 NC spleen	RFC per 10^5 NC
		g								
1‡ (18)§	1	1.27	3	16	267	NA		107	89	12
2 (18)	1	1.40	2	41	225	6.30	0.01	410	113	36
	2	1.38	2	14	168			140	84	17
3 (8)	1	1.29	3	14	211	NA		93	70	13
4 (8)	1	1.35	3	25	432	NA		167	144	12
5 (8)	1	1.23	3	18	381	NA		120	127	9
6 (8)	1	1.18	3	5	241	40.14	10^{-9}‖	33	80	4
	2	1.17	3	39	241			260	80	32
	3	1.25	3	14	433			93	144	6
7 (8)	1	1.27	3	7	184	2.86	0.09	47	61	8
	2	1.23	3	4	299			27	100	3
8 (8)	1	1.26	3	21	192	10.64	0.06	140	64	22
	2	1.30	3	17	293			113	98	12
	3	1.29	3	13	178			87	59	15
	4	1.25	3	11	165			73	55	13
	5	1.25	3	27	207			180	69	26
	6	1.16	3	7	139			47	46	10
9 (26)	1	1.20	2	25	137	5.16	0.08	250	69	36
	2	1.22	3	29	277			193	92	21
	3	1.17	3	17	187			113	62	18
10 (8)	1	1.22	1	5	222	4.90	0.09	100	222	5
	2	1.22	6	12	512			40	85	5
	3	1.17	6	25	537			83	90	9
11 (18)	1	1.16	3	6	146	6.40	0.17	40	49	8
	2	1.26	3	13	259			87	86	10
	3	0.96	3	2	121			13	40	3
	4	1.18	3	14	174			93	58	16
	5	1.27	3	17	316			113	105	11
12 (18)	1	1.16	3	20	186	0.23	0.89	133	62	22
	2	1.16	3	15	165			100	55	18
	3	1.25	3	21	217			140	72	19
13 (18)	1	1.27	3	34	281	0.52	0.47	227	94	24
	2	1.17	3	21	214			140	71	20
14 (18)	1	1.16	3	13	262	34.91	10^{-7}¶	87	87	10
	2	1.07	3	11	132			73	44	17
	3	1.34	3	56	312			373	104	36
	4	1.35	3	24	493			160	164	10
Mean		1.229						126.9	86.4	15.3
Standard deviation		0.085						87.0	36.8	9.2

* Test of homogeneity within each litter.

‡ Not made blind; all other cell counts in this table were made blind.

§ The number in parentheses indicates the sheep whose erythrocytes were used.

‖ It proved impossible to obtain convergence in attempting to fit an affine model to this litter.

¶ Not adequately fitted by an affine linear model.

mated n between parental strains in number of TNP-SRBC RFC per 10^5 nucleated cells was 0.12 from equation (1) and 0.06 from equation (2), i.e., not large and probably less than 10 loci. The estimates using the square-root of TNP-SRBC RFC per 10^5 nucleated cells were 0.16 from equation (1) and 0.07 from equation (2), and again were not large.

Relationship between Repertoire Size and Repertoire Composition in Hybrid Mice. To test the independence of the controls on the size of the repertoire

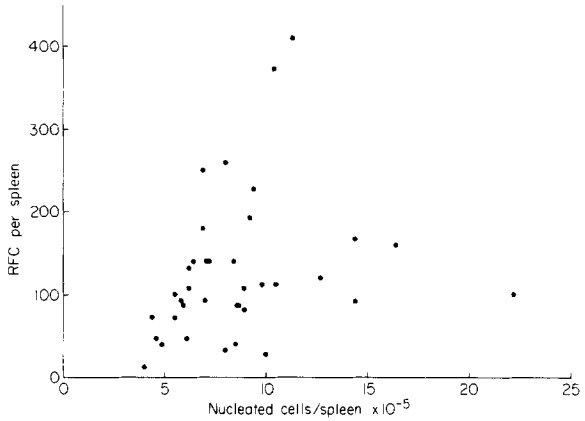

FIG. 3. Numbers of TNP-specific RFC in the spleens of BALB/c × (BALB/c × CBA/J) backcross fetal mice (Table V) in relation to spleen size, as in Fig. 1. Each point represents the results obtained for one spleen. All fetuses were tested on the 18th day of gestation.

TABLE VI

Numbers of TNP-SRBC RFC and Nucleated Cells in the Spleens of Individual CBA/J × (BALB/c × CBA/J) Backcross 18-Day Fetal Mice

Litter serial number	Fetus serial number	Fetal weight	Observed sample counts*			Calculated estimates		
			Aliquots	TNP-SRBC RFC	NC	RFC per spleen	10^4 NC per spleen	RFC per 10^5 NC
		g						
1	1	1.04	4	28	205	140	51	27
	2	1.04	4	6	95	30	24	13
	3	1.04	2	15	92	150	46	33
	4	0.99	4	26	115	130	29	45
	5	0.93	4	20	143	100	36	28
Mean		1.008				110.0	37.1	29.1
Standard deviation		0.049				48.5	11.5	11.7

* All cell counts in this table were made blind using RBC from sheep 26. A test of homogeneity within this litter gave $G^2 = 7.44$ with four df, $P = 0.11$.

(measured as the proportion of TNP-SRBC RFC to nucleated cells) from controls affecting the composition of the repertoire (measured as the ratio of proportions of cells specific for different antigens), the frequencies of cells specific for each of two antigens were measured for individual BALB/c × (BALB/c × CBA/J) fetal spleens. In two fetuses in one litter, and in a third fetus in another litter, the spleens were split and assayed for the proportions of RFC with TNP-SRBC and SRBC (obtained from sheep 18). Although the proportion of TNP-SRBC RFC to nucleated cells varied significantly within this group of three fetuses ($G^2 = 15.6$ with two df, $P < 0.0005$), the ratio of proportions (TNP-SRBC RFC/nucleated cells)/(SRBC RFC/nucleated cells) was the same for the three individuals and

TABLE VII

Numbers of TNP-SRBC RFC and Nucleated Cells in the Spleens of Individual (BALB/c × CBA/J) × (BALB/c × CBA/J) F_2 18-Day Fetal Mice

Litter serial number	Fetus serial number	Fetal weight	Observed sample counts			Calculated estimates		
			Aliquots	TNP-SRBC RFC	NC	RFC per spleen	10^4 NC per spleen	RFC per 10^5 NC
		g						
1*	1	1.25	3	8	285	53	95	6
	2	1.11	3	3	73	20	24	8
	3	1.07	3	2	141	13	47	3
	4	1.14	3	2	216	13	72	2
2	1	1.64	2	63	195	630	98	65
Mean		1.242				146.0	67.2	16.6
Standard deviation		0.232				271.1	31.5	26.9

* All cell counts in this table were made blind using RBC from sheep 8. A test of homogeneity within litter 1 gave $G^2 = 3.78$ with three df, $P = 0.29$. For all F_2 fetuses, $G^2 = 116.5$ with four df, $P < 10^{-20}$.

indistinguishable from the same ratio of proportions computed for inbred CBA/J and BALB/c fetuses using sheep 7 RBC (Table VIII); the ratios of proportions differed from those of the ancestral strains using sheep 26 RBC. The relative proportions of cell populations binding specifically to the antigens tested in these backcrosses were indistinguishable from the same relative proportions in the ancestral inbred strains, despite the differences observed between backcross and inbred individuals in the sizes of these cell populations relative to total spleen size.

Discussion

The process by which antigen-specific cells are generated in the fetal mouse is known to be subject to stringent controls (1–3, 10–12). Most of the developmental controls described to date do not affect the generation of the antigen-specific cells themselves, however, but rather appear to regulate the subsequent interactions of these cells to generate a full immune response (13–15).

The data presented here are novel in two respects. First, they suggest the existence of two sets of controls that affect directly the generation of antigen-binding cells: one affects the proportions of different antigen-binding cells within the population of committed lymphocytes (i.e., the composition of the repertoire), and the other affects the total size of that repertoire within the fetal spleen. The second novel aspect of these data is that, at least for CBA/J and BALB/c mice, both of the controls appear to function independently of maternal or environmental effects, within the range of environmental variation in our breeding colonies. In particular, no more variation in the composition and size of the repertoire is seen from individual to individual within a strain than would be expected in replicate random samples drawn from a homogeneous population

TABLE VIII

Numbers of RFC for Two Different Antigens and Nucleated Cells in the Spleens of Individual BALB/c × (BALB/c × CBA/J) 18-Day Fetal Mice

Litter serial number	Fetus serial number	Fetal weight	Observed sample counts*						Calculated estimates‡			TNP-SRBC RFC per sheep 18 RBC-RFC
			Aliquots	TNP-SRBC RFC	NC	Aliquots	SRBC-RFC	NC	TNP-SRBC RFC per spleen	Sheep 18 RBC RFC per spleen	10^5 NC per spleen	
		g										
1	1	1.27	3	16	267	3	2	280	107	13	9	8.0
2	1	1.40	2	41	225	4	8	450	410	40	11	10.3
	2	1.38	2	14	168	4	3	441	140	15	10	9.3
Mean		1.35							219	23	10	9
Standard deviation		0.07							166	15	1	1

* Sheep 18 RBC were used as TNP carrier and as antigen. NC, nucleated cells.

‡ Homogeneity of ratio of ratios [(TNP-RFC)/NC]/[(SRBC-RFC)/NC] for these three fetuses $X^2 = 0.15$ with two df, P = 0.93. Homogeneity for these three fetuses and CBA/J and BALB/c cell counts, pooled within each inbred strain, using sheep 7 RBC, $X^2 = 6.29$ with four df, $P = 0.18$; same test using sheep 26 RBC with inbred strains, $X^2 = 25.13$ with four df, $P = 10^{-4}$.

of cells in a single individual. This finding is in agreement with the requirement of the clonal selection theory (16) that the repertoire of antigen-binding cells arise independently of any interaction with foreign antigens. However, the precision of control revealed by the present observations is not predicted by the clonal selection theory.

The two sets of controls are best defined in terms of the patterns seen in the antigen-binding cell populations of CBA/J and BALB/c fetal mice. Their independence of one another can be shown by comparing these data with the data obtained for Swiss-L fetal mice. Within the inbred CBA/J and BALB/c mouse strains, in fetuses at 18 days of gestation, the number of nucleated spleen cells that bound the hapten antigens TNP and Tosyl and the surface antigens of native SRBC of different individual sheep was a constant proportion of the total number of nucleated spleen cells. The ratio of the proportion of nucleated spleen cells specific for a given antigen in CBA/J mice to the proportion in BALB/c mice of nucleated spleen cells specific for the same antigen equaled, within sampling fluctuation, the ratio of the proportion in CBA/J mice of nucleated spleen cells which had detectable immunoglobulin on their surface to the proportion of such cells in BALB/c mice (Table II). Because the frequency of splenic T cells in these mice is low at this stage of development, this observation suggests that the nucleated cells of the fetal spleen may be viewed as falling into two subpopulations, or compartments, those bearing detectable immunoglobulin on their surface (Ig+ cells) and those without detectable immunoglobulin on their surface. Within the compartment of Ig+ cells, the fraction of cells specific for a given antigen appears to be the same for both of the two inbred strains examined, suggesting that the regulation of compartment size relative to total nucleated cells per spleen may be independent of the regulation of compartment composition (Table II).

This conclusion is supported by the analysis of antigen-binding cell populations in outbred Swiss-L fetal mice. While the absolute and relative frequency of

TNP-specific cells varied significantly from fetus to fetus, the ratio of proportions of cells specific for two different antigens was constant within sampling fluctuation for all the fetuses tested. This observation suggests variation in repertoire size among Swiss-L individuals, while repertoire composition remained constant. At the same time the ratio of proportions observed for the two antigens in Swiss-L fetuses differed from the ratio in the inbred strains. Hence the methods used for counting specific antigen-binding cell populations are sensitive enough to detect differences in the composition of the Ig+ cell compartment, and suggest that the control of repertoire composition is not invariant for all strains of mice.

While controls directly affecting the size of antigen-binding cell populations have not been reported previously, several controls affecting other quantitative aspects of splenic lymphoid cell populations have been observed that are consistent with the findings. At 2 wk postpartum the frequency of splenic B cells expressing surface complement receptors varies significantly from strain to strain of inbred mouse. Within a strain, the amount of variation in this frequency is greatly reduced (17). The absolute number of these cells, however, appears to be constant even between strains (18). Similarly, for at least one pair of strains, the frequency of splenic T cells 2 wk after birth differs significantly, but the number of these cells is the same for both strains (18). These observations suggest that the sizes of the B- and T-cell populations in the spleen are tightly controlled in young inbred mice.

The observation that the proportion of antigen-specific cells to nucleated cells was invariant within a strain, but differed sharply between strains, raised the possibility that this relative frequency was genetically determined. Comparison of the proportions of TNP-specific cells in the spleens of F_1 and F_2 hybrids, and backcross fetuses, to those observed in fetuses of the parental strains indicated that this was the case, and further suggested that the frequency was determined by the action of one or a few closely linked autosomal Mendelian genes. In addition, these data indicate that the genetic factors in CBA/J mice controlling relative rates of increase of RFC are dominant to those in BALB/c mice.

In most of the hybrid fetuses, the number of RFC was proportional to the excess in the number of nucleated cells over a threshold, rather than, as in the inbred strains, directly proportional to the total number of nucleated cells. The threshold may indicate a delayed start in the expansion of the lymphoid compartment of the spleen, relative to the nonlymphoid compartment.

These genetic experiments suggest that different genetic factors influence the fraction of fetal spleen cells which bear immunoglobulin on their surfaces, and the proportions among those Ig+ cells of specific antigen-binding RFC, and that both of these factors are independent of factors influencing body weight. The genetic controls on the repertoire composition operate with such precision that by the time there are roughly 15,000 or 2^{14} Ig+ cells in the fetal mouse spleen, the proportions of Ig+ cells binding specifically to each of various antigens in different individuals of a given genetic background fluctuate no more than could be explained by sampling error.

Obviously, general conclusions concerning the development of antigen-specific cells must be qualified by the fact that the number of antigens tested so far

is still an extremely small fraction of the total number of antigens. It nevertheless seems likely that a full range (although perhaps not a full density) of antigen-binding cells is already specified by the 18th day of gestation.

It thus becomes necessary to explain how such a wide repertoire is generated in the course of fetal development. Three factors might be expected a priori to be directly involved: the genes coding for Ig variable regions, inherited by the fetus in its germ-line genome; somatic mutational and recombinational mechanisms acting in the developing fetus to generate a larger set of genes from the germ-line sequences; and selective mechanisms, acting negatively or positively to alter the relative frequencies of antigen-binding specificities ultimately expressed in the repertoire.

A simple germ-line theory would suppose that each different antigen-specific cell expresses a different germ-line gene and that the proportions observed in the antigen-binding cell repertoire result from the regulated expression of these genes (19). While this theory provides a ready qualitative explanation for the control of repertoire composition, recent experiments suggest that it is quantitatively inadequate to account for the diversity of specificities in the repertoire. In particular, the number of germ-line genes now appears to be as small as one per haploid genome per heavy or light chain subgroup in at least some cases (20, 21).

It therefore appears that many, perhaps most, of the genes coding for the full repertoire of Ig variable regions are generated by somatic mutation or related alterations in germ-line DNA. Within this framework, our data indicate that the mechanism which generates the repertoire must yield reproducible proportions of antigen-binding specificities in the absence of selection by antigens other than those ordinarily present in the fetal mouse, and that this mechanism may function in as few as 14 generations of cell division. These data appear incompatible with any theory which supposes that positive selection by antigens outside of the individual fetus determines the composition of the repertoire of antigen-binding specificities. In principle, however, this remarkably precise and early control of the repertoire could be explained by the action of negative selection on the repertoire generated by somatic mutation or recombination.

Nossal and Pike (22) have proposed, for example, that an immature lymphocyte exposed to foreign antigen is immediately and irreversibly stripped of its surface Ig. Such a cell would not be detected either as an Ig+ cell or as an antigen-binding cell in experiments of the sort described here. If this negative selection were imposed by an invariant panel of self-antigens, such as the developing animal's own histocompatibility antigens (23), certain clones would be completely absent from the animal's repertoire at all stages of development. A constant negative selective force of this sort would cause no detectable maturation of the repertoire, either in the range of antigen-binding specificities expressed, or in the distribution of avidities with which any particular foreign antigen is bound. Such negative selection would be expected to lead to fine differences in the antigen-binding repertoires expressed by genetically distinct strains of mice. Within the limits of precision of the assays used, however, the present observations detect no difference in the composition of the CBA/J and BALB/c fetal repertoires in spite of the differences between the two strains in a variety of genetic markers including *H-2* (24).

Our data suggest that the repertoire may be generated rapidly and somatically, and that it arises largely in the absence of positive or negative selection for cells of particular antigen-binding specificities. It is possible to construct models of somatic mutation compatible with these restrictions and with the observed constancy of the repertoire composition from individual to individual (J. E. Cohen, P. D'Eustachio, and G. M. Edelman, unpublished observations). It will be of considerable interest to determine whether the frequency and distribution of immunoglobulin variable region sequences generated by such models accurately reflect the patterns seen in the sequences of actual Ig's, and to test the predictions of these models against further studies of fetal and adult lymphocytes and their precursors.

Summary

In order to analyze the genetic and physiological basis of controls affecting the generation of the repertoire of antigen-binding cells in fetal mice, we have measured the numbers of spleen cells specific for each of four antigens as a function of the total numbers of nucleated and Ig-bearing cells in inbred, hybrid, and random bred fetuses. For each of the two inbred strains BALB/c and CBA/J, the proportion of nucleated cells specific for a given antigen was the same for all individuals of the strain at the 18th day of gestation. The proportion did vary from antigen to antigen, however, and for each antigen the proportion of specific cells observed in CBA/J fetuses was approximately four times that observed in BALB/c fetuses. This difference appeared to be due to a difference between the two strains in the relative size of the repertoire of antigen-binding spleen cells at this stage of development, inasmuch as the frequency of Ig-bearing spleen cells in CBA/J fetuses was likewise approximately four times that observed in BALB/c fetuses.

In random bred Swiss-L fetal mice at the 18th day of gestation, the proportion of cells specific for a given antigen varied significantly from one individual to the next. The ratio of proportions of the two antigens observed was constant from individual to individual, however, and this constant ratio differed significantly from the ratio observed for the same two antigens in fetal BALB/c and CBA/J inbred mice. These data suggest that the ontogeny of the repertoire of antigen-binding cells in fetal mice is subject to at least two independent sets of controls, one affecting the relative size of the repertoire in the spleen, and the other affecting the distribution of antigen-binding specificities within that repertoire.

Analysis of repertoire size and composition in the spleens of hybrid fetuses confirmed the observation that the two parameters are controlled independently, and suggested further that the control of repertoire size in these fetuses is due to the action of one or a few closely-linked autosomal Mendelian genes. These data are consistent with models for the origin of antibody diversity in which the genes coding for the full repertoire of antibodies are generated somatically from a small number of germ-line genes early in development and in the absence of any strong positive or negative selection with respect to antigenic specificity.

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