VARIATION AND CONTROL OF SPECIFIC ANTIGEN-BINDING CELL POPULATIONS IN INDIVIDUAL FETAL MICE*

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During development, the embryonic mouse generates a repertoire of lymphoid cells capable of specifically recognizing a large range of different foreign antigens. Previous studies have suggested that, within the limits of precision of the assays used, the repertoire of cells capable of specifically binding different antigens is filled early and uniformly, and that this process occurs in the absence of selection by foreign antigens (1, 2).

Measurements of the numbers of antigen-binding cells in the spleens of individual outbred fetal mice did not reveal subpopulations of individuals differing systematically from the fetal population as a whole. Nevertheless, significantly more variation was found among individuals than would be expected from sampling fluctuation, assuming that the actual number of cells binding a specific antigen is constant, or nearly so, among fetuses (2).

To determine the source of this variation more precisely, we have now measured the numbers of cells specific for each of two antigens, trinitrophenyl (TNP) and sheep red blood cells (SRBC), in the spleens of individual random-bred Swiss-L and inbred CBA/J and BALB/c fetal mice. These numbers were then related to spleen size as estimated by counts of total nucleated cells, and the results were subjected to stringent statistical tests. For outbred Swiss-L fetuses, the ratio of antigen-binding cells to nucleated cells varied significantly more than could be explained by sampling fluctuation. For each inbred strain, however, the number of cells specific for a given antigen was a constant proportion of the number of nucleated cells. 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, differed no more from CBA/J to BALB/c mice than would be expected in repeated samples of cells from the spleen of a single fetus.

These results indicate that the development of antigen-binding cells in the individual fetus conforms to the uniform pattern seen for populations of fetuses. The precision with which these antigen-binding cell populations proliferate suggests further that the development of these cells may be subject to strong genetic controls.

Materials and Methods

Detection of Antigen-Binding Cells. Spleens were removed from individual outbred Swiss-L and inbred CBA/J ($H\text{-}2^k$) and BALB/c ($H\text{-}2^d$) fetal mice on the 18th day of gestation. Single cell

* This work was supported by U. S. Public Health Service grants AI 11378 and AI 09273 from the National Institutes of Health to G. M. E.; and by National Science Foundation grant BMS74-13276 to J. E. C.

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suspensions were prepared and assayed for cells capable of binding SRBC or TNP-derivatized SRBC (TNP-SRBC) to form rosettes as described previously (2). SRBC from three individual sheep (Microbiological Associates, Bethesda, Md.; sheep nos. 5, 7, and 26) were used in all these experiments; the red cells were stored at 4°C and used within 10 days of removal from the sheep. For every fetus, the number of 25-μl aliquots of the final cell suspension counted for rosette-forming cells (RFC) was equal to the number of 0.05-μl aliquots counted for nucleated cells, so that the ratios, RFC counted:nucleated cells counted, were comparable from one fetus to another. In experiments done "blind," the labels on the tubes containing the cell suspensions were changed after the RFC had been counted and before the nucleated cells were counted; the experimenter learned the identity of the fetus from which any given sample of cells came only at the end of the experiment.

Statistical Methods. To determine whether the variation in the numbers of RFC from fetus to fetus exceeded the variation expected from sampling fluctuation alone, the Poisson variance test was used (3). To determine whether the ratio, RFC: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 proportions which would be expected if the ratio were the same for all the fetuses in the table. Goodness of fit was measured by the G^2 statistic (4). To determine whether the ratio of proportions of TNP-specific RFC and SRBC-specific RFC differed from one strain to another, the method of Fleiss (5) was used. In all cases, the statistical analyses were performed using the numbers of cells actually observed, rather than estimates derived from these numbers (e.g., total RFC per spleen).

Results

The variation in the numbers of RFC found in the spleens of random-bred Swiss-L fetal mice has been reported previously (2). In 81 spleens tested for TNP-specific RFC, the mean and standard deviation of the actual numbers of RFC observed in aliquots equivalent to $^1/_{20}$ of the nucleated cells in the spleen were 12.53 and 9.61. In a single litter of 10 fetuses taken from this population, the mean and standard deviation were 9.90 and 6.03. In either case, the probability that such large variation in numbers of RFC could have arisen because of fluctuations due to sampling from fetal cell populations with constant numbers of RFC is less than 10^{-3}.

The estimated total numbers of nucleated cells in these spleens were themselves highly variable, however, ranging from 3.5×10^5 to 17.0×10^5. To test the possibility that the fluctuation in the RFC number reflected these differences in the sizes of individual fetal spleens, the number of TNP-specific RFC and the number of nucleated cells were determined for each of 45 Swiss-L fetuses from nine different litters (Fig. 1). If the ratio, total RFC:total nucleated cells, were constant from one fetal spleen to another, the results of these assays would have fallen along a straight line. This was not the case, and as much variation in the ratio was observed within as between litters.

Fig. 2 shows the results obtained when fetuses of the inbred strains BALB/c and CBA/J were assayed. The ratio of RFC to nucleated cells was nearly constant for each strain and each antigen. The clear differences between strains and between antigens were confirmed by statistical analysis of the original cell counts. All the data, and a detailed analysis of data for CBA/J fetuses tested for TNP-specific RFC, are given in Table I. Within each of the inbred strains CBA/J and BALB/c and for each of the antigens TNP-SRBC and plain SRBC, the ratio of RFC to nucleated cells varied no more than would the ratio in repeated

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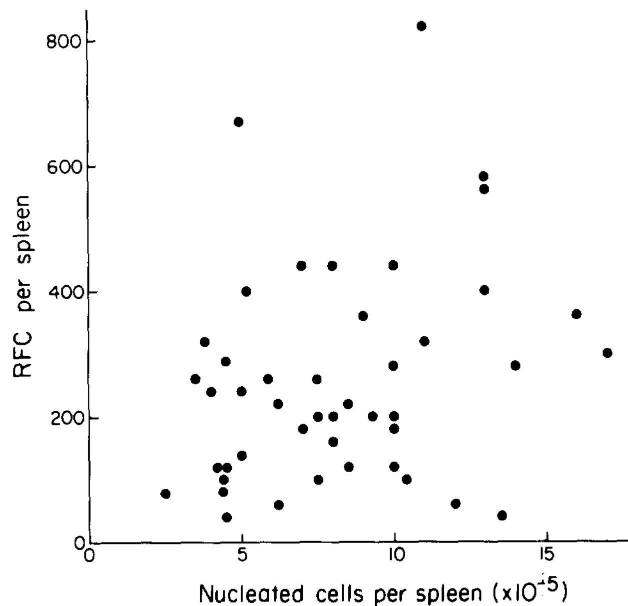

FIG. 1. Numbers of TNP-specific rosette-forming cells in the spleens of Swiss-L fetal mice as a function of spleen size. RFC number per spleen was calculated from the number of RFC observed in 25-μl aliquots of the rosette assay mixture (equivalent to $^1/_{20}$ spleen). The number of nucleated cells 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.

samples from a single fetal spleen. The ratios did, however, differ significantly between strains and antigens.

For each antigen, the RFC:nucleated cell ratio was higher in the CBA/J mice than in the BALB/c mice (Table II). Surprisingly, the ratio of the number of TNP-SRBC RFC to the number of SRBC RFC in a fixed number of nucleated cells was the same for both strains, within sampling fluctuation.

Discussion

Analysis of the proportions of RFC and nucleated cells in the spleens of individual 18-day fetal mice indicates that although these proportions were nearly constant for any given antigen and strain of inbred mice, they differed significantly from strain to strain and from antigen to antigen. The ratio of the proportions for the two antigens, however, was the same for both strains.

Within either of the inbred strains CBA/J and BALB/c, for the antigens SRBC or TNP-SRBC, the ratio of the number of antigen-binding cells (detected here as RFC) to the number of nucleated cells varied no more than would be expected in repeated samples of a cell suspension prepared from a single fetus. This fact demonstrates the high reproducibility of the experimental procedure for preparing and counting cells. It also establishes that, within each of the two strains, the relative sizes of the RFC populations specific for TNP-SRBC and for plain SRBC do not vary from one fetus to another. If there were such individual variation, there would be detectable fluctuation in the RFC:nucleated cell ratio from fetus to fetus within a strain for at least one of the antigens.

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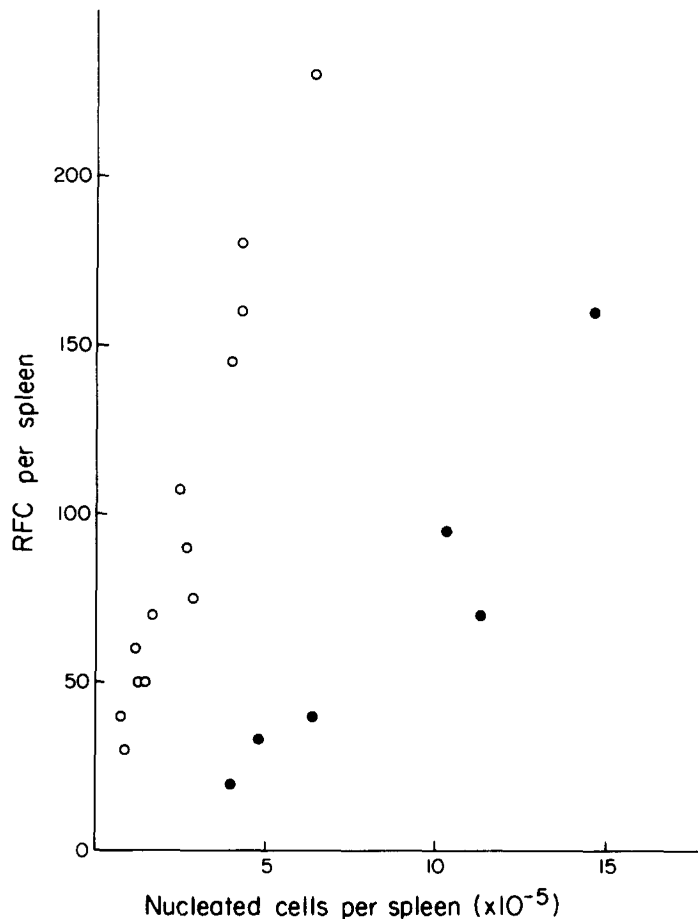

FIG. 2. Numbers of TNP-specific rosette-forming cells in the spleens of individual CBA/J (○) and BALB/c (●) fetal mice as a function of spleen size. All fetuses were tested on the 18th day of gestation.

The differences in the RFC:nucleated cell ratios between strains and between antigens are both statistically significant and quantitatively large. The ratio of the number of TNP-SRBC-specific RFC to the number of SRBC-specific RFC in a fixed number of nucleated cells from the spleen of a fetal CBA/J mouse, however, was equal to the corresponding ratio for a fetal BALB/c mouse, within the range of variation which would arise from sampling alone. This unexpected result obviously must be checked and confirmed for a variety of different antigens, but if it is generally valid, it suggests that the proportions of cells specific for different antigens in the developing immune system are tightly controlled. The control of the proportions of cells of various types has been remarked in organisms ranging from slime molds (6) to mice (7). The novelty in our findings is the demonstration of such quantitative control in cell populations of the immune system, based on actual cell counts from individual fetuses.

The homogeneity within inbred strains and the differences between them (Fig. 2) suggest that the variability evident in the Swiss-L fetuses (Fig. 1) is associated in some way with the genetic heterogeneity of these random-bred

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TABLE I

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

Litter serial number	Fetus serial number	Observed sample counts*			Calculated estimates		
		Aliquots	RFC	Nucleated cells	RFC per spleen‡	10^4 n. c. per spleen§	RFC per 10^6 n. c.
Antigen: TNP-SRBC							
Mouse strain: CBA/J							
1 (26)‖	1	2	23	128	230	64	359
2 (26)	1	2	18	86	180	43	419
	2	2	16	85	160	43	376
	3	2	7	33	70	17	424
	4	2	9	54	90	27	333
3 (26)	1	4	10	59	50	15	338
	2	4	8	32	40	8	500
	3	4	6	34	30	9	353
4 (26)	1	4	29	159	145	40	364
	2	4	15	116	75	29	259
5¶ (5)	1	2	5	26	50	13	385
	2	2	6	24	60	12	500
6¶ (26)	1	3	16	75	107	25	427
Mouse strain: BALB/c							
7 (26)	1	6	12	381	40	64	63
	2	6	10	289	33	48	69
8¶ (5)	1	2	7	226	70	113	62
	2	2	2	80	20	40	50
9¶ (5)	1	4	19	412	95	103	92
	2	4	32	582	160	146	110
Antigen: SRBC							
Mouse strain: CBA/J							
10 (7)	1	8	4	42	10	5	189
11 (7)	1	6	4	159	13	27	50
12 (7)	1	6	6	112	20	19	107
	2	2	0	26	0	13	0
13¶ (7)	1	6	5	157	17	26	63
Mouse strain: BALB/c							
14¶ (7)	1	6	7	817	23	136	17
	2	6	5	448	17	75	22
15¶ (6)	1	5	5	296	20	59	34
	2	5	4	416	16	83	19
16 (6)	1	5	3	550	12	110	11

* There was no significant heterogeneity in the RFC/nucleated cell ratio within litter 2 ($G^2 = 0.326$ with 3 degrees of freedom [df]), litter 3 ($G^2 = 0.596$ with 2 df), litter 4 ($G^2 = 1.044$ with 1 df), or litter 5 ($G^2 = 0.155$ with 1 df). Using the counts pooled within litters, there was no significant heterogeneity among litters 1-6 ($G^2 = 1.481$ with 5 df). For all these tests, $P > 0.25$. Similar hierarchical analysis of within- and between-litter variation in the remaining observations showed no significant variation between individuals for any combination of antigen and strain.

‡ RFC/spleen = sampled RFC × 20/aliquots.

§ 10^4 nucleated cells (n. c.)/spleen = sampled n. c./aliquots.

‖ Number in parenthesis indicates the sheep whose blood cells were used, plain or derivatized with TNP. The homogeneity of results for TNP-SRBC assays using cells from different sheep indicates that the antigen detected in these assays is TNP plus those determinants common to all SRBC.

¶ Counted blind.

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TABLE II

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

Mouse strain	TNP-SRBC RFC per 10^5 n. c.*	SRBC RFC per 10^5 n. c.	TNP-SRBC RFC/SRBC RFC
CBA/J	36.88 ± 6.07‡	7.66 ± 2.77	4.81 ± 1.91§
BALB/c	8.32 ± 2.88	1.90 ± 1.38	4.38 ± 3.52

* n. c., nucleated cells.

‡ Mean ± standard deviation per 10^5 cells. Mean = 200 × RFC/n. c., where RFC and nucleated cells are the sums of the corresponding counts in Table I for all individuals of the given strain tested for the given antigen. Standard deviation = $(\text{mean})^{1/2}$, based on Poisson approximation to the binomial distribution.

§ Ratio of means from Table II ± approximate standard deviation of ratio of independent variates, neglecting the possibility of 0 RFC. Analysis of data in Table I using method of Fleiss (5) indicates that variation of (TNP-SRBC RFC/n. c.)/(SRBC RFC/n. c.) between CBA/J and BALB/c fetuses is within the range expected from sampling alone: $\chi^2 = 0.076$ with 1 degree of freedom, $P > 0.75$.

mice. Genetic control of the immune system in mice has heretofore been analyzed most successfully by programs of artificial selection of lines of animals showing heritable low or high levels of immune responsiveness to complex immunogens. At least four independently assorting genes have been shown to contribute to the "high" or "low" responder phenotype, by affecting such factors as the efficiency with which the animals' macrophages process foreign antigens (8). The experiments described in this paper provide the first evidence suggesting genetic controls that affect the repertoire of antigen-binding cells itself. Furthermore, preliminary cross-breeding experiments suggest that the difference observed between CBA/J and BALB/c fetuses may be due to the effects of only one or a few genes.

It would be entirely consistent with our findings if the differences in the frequency of RFC observed between fetuses of the two inbred strains were due simply to a difference in the proportions of antigen-specific lymphoid cells in their spleens at this particular stage of fetal development. This possibility can be tested directly, inasmuch as it would give rise to differences in the proportions of immunoglobulin-bearing cells in the spleens of individual fetuses of each strain. Confirmation of this possibility would be compatible with the hypothesis that the generation of antigen-binding specificities within the population of developing lymphoid cells proceeds by the same mechanism in CBA/J and BALB/c mice.

It seems unlikely, however, that a small number of structural genes would alone be sufficient to specify precisely both the composition of the repertoire of antigen-binding specificities and the processes by which the repertoire is expressed in the course of development. Other more general mechanisms may be necessary to explain the control of cell populations in the developing immune system. A number of models seem plausible. These include selective action on the development of clones of lymphoid cells due to the presence of antigens from other tissues in the organism; direct interactions of lymphoid cells via their surface receptors; interactions of lymphoid cells via diffusible substances specific to different cell types; and even certain stochastic models (multitype branching processes) in which cell lines proliferate independently of one another. Further experimental investigation of these models will require extension of the present studies to other antigens and to other mouse strains with differences in *Ir* genes and in those coding for H-2, Ia antigens, and immunoglobulin allotypes.

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Summary

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 were measured as a function of spleen size. For Swiss-L fetuses, the ratio of antigen-binding cells to nucleated cells varied more than would arise 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, differed no more from CBA/J to BALB/c mice than would be expected in repeated samples of cells from the spleen of a single fetus. These results confirm at the level of the individual fetus the uniform pattern of development seen for populations of fetuses. They reveal a surprising precision in the proliferation of specific antigen-binding cell populations and suggest that the development of these cells may be subject to strong genetic controls.

Received for publication 12 April 1976.

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