cubation of CRL with antiserum to θ and guinea pig complement does not interfere with their subsequent capacity to bind EAC. Similar results were obtained when complement was omitted from the incubation medium; that is, we were unable to inhibit rosette formation between EAC and CRL (from AKR mice) by prior treatment of the lymphocytes with antiserum to θ (AKR) for 45 minutes at 37°C.

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8 March 1971; revised 17 May 1971

Density Gradient Separation of Marrow Precursor Cells Restricted for Antibody Specificity

Abstract. *Potentially immunocompetent cells of* $(C57BL/6 \times DBA/2)F_1$ *mouse bone marrow are committed to antigenic determinants of sheep or burro erythrocytes prior to interaction with thymus-derived cells and participation in immune responses to administered antigens. At this stage of differentiation marrow cells of this particular mouse strain are not yet restricted for the immunoglobulin M or immunoglobulin G antibody class. By equilibrium centrifugation in discontinuous gradients of bovine serum albumin, precursors of cells that produce antibody to sheep erythrocytes migrate to denser regions, whereas the precursors of immunocytes that produce antibody to burro erythrocytes remain in the lower density regions.*

Recent evidence suggests that primitive precursors of antibody-forming cells, which are marrow derived in mice, are predetermined for the specificity of antibody to be synthesized by descendant immunocytes (*1*). This evidence was obtained by specifically paralyzing or sensitizing marrow-derived cells before interaction with thymus-derived and macrophage-like accessory cells. In addition, the population of marrow precursors undergoes long-term changes on exposure to antigens that specifically enhance immunological competence, while terminal differentiation and maturation remain dependent on accessory cells (*2*). Thus, the role of cellular interactions is not likely to be the transfer of information for antibody specificity from thymic cells to precursors of immunocytes, and marrow-derived cells could be the carriers of immunological memory.

In most mouse strains, marrow cells are predetermined also for the molecular class of descendant immunocytes, as demonstrated by limiting dilution analysis (*3*) and, more directly, by physical separation of unipotent cells (*4*). The only known exception occurs in $(C57BL/6 \times DBA/2)F_1$ mice whose marrow cells are pluripotent, according to limiting dilution experiments, for hemolytic antibodies to sheep erythrocytes (SRBC) which are of the IgM and IgG classes (*3*). We now present direct evidence confirming the lack of class restriction of $(C57BL/6 \times DBA/2)F_1$ marrow cells engaged in immune responses to SRBC (anti-sheep) and extending the finding to cells engaged in responses to burro erythrocytes (BRBC) (anti-burro). In addition, a second property of these cells is described, namely, the restriction for specificity of antibodies.

Bone marrow cells, harvested from the long bones of 12-week-old female mice, were suspended in Eagle's medium and subjected to equilibrium centrifugation in a discontinuous gradient of 17 to 35 percent bovine serum albumin (BSA), according to the method of Dicke *et al.* (*5*), as described earlier (*4*). Ten cell fractions were collected from the interphases beginning at the top between 17 and 19 percent BSA. The fractions were diluted in Ca- and Mg-free phosphate-buffered (*p*H 7.2) saline (PBS), washed once by centrifugation, and resuspended in PBS. Nucleated cells were counted with an electronic particle counter (*6*). Density distributions of nucleated cells have been described (*4*). The profiles were reproducible and displayed a major peak in the denser region of the gradient (29 to 35 percent BSA). Recovery of nucleated cells after centrifugation ranged from 65 percent to 73 percent of input in the three experiments described below.

To test for potentially immunocompetent cells, each marrow fraction was added to an equal volume of thymocyte suspensions, and the mixtures were transplanted by way of the tail vein into each mouse of several groups of syngeneic female mice that were heavily irradiated (*7*). Each group contained 10 to 20 animals, and each inoculum contained a limiting number of 2.5×10^5 bone marrow cells (fractionated or not) and an excess number of 4×10^7 thymocytes (*8*). Eighteen hours after transplantation, one-half of the recipients received an intravenous injection of 5×10^8 washed SRBC, and the other half received 5×10^8 washed BRBC. Direct (IgM) and indirect (IgG) plaque-forming cells (PFC) releasing hemolytic antibody were enumerated in cell suspensions of recipient spleens 9 days after transplantation by the agarose-slide technique (*9*). Spleen cells of recipients immunized against SRBC were assayed for anti-sheep PFC, and those of recipients immunized against BRBC for anti-burro PFC. Animals were classified as "positive" or "negative," depending on whether their spleens contained PFC in numbers greater than or equal to those of controls. Negative controls were irradiated mice inoculated with marrow cells and thymocytes but not with SRBC or BRBC, and mice inoculated with marrow cells (fractionated or not) and antigens but not with thymocytes. The numbers of PFC in spleens of these controls did not exceed 200 direct and 100 indirect. In positive spleens, the numbers of PFC ranged from 200 to

Table 1. Chi-square tests for independence of antibody responses of the IgM and IgG classes (direct and indirect PFC) in mice injected with fractionated marrow cells, thymocytes, and either SRBC or BRBC. Mice injected with marrow cells of fractions 27 to 31 percent BSA were included in the test for anti-sheep responses, and mice injected with cells of fractions 23 to 29 percent BSA for anti-burro responses. Chi-square values in the table were compared with 7.88, the critical value of chi-square statistic at the .005 level of significance; +, recipient spleens with > 200 direct or 100 indirect PFC; −, spleens with fewer PFC.

Direct PFC	Indirect PFC	Anti-SRBC (No.)	Anti-BRBC (No.)
+	+	48	49
+	−	8	5
−	+	0	2
−	−	11	10
χ^2		29	26

2000 direct and from 100 to 1700 indirect. As judged by the PFC assay, antibody produced in recipients of marrow-thymus cell mixtures stimulated with SRBC did not react with BRBC, and vice versa. Pooled results of three experiments are shown in Fig. 1.

In each experiment, a large proportion of mice injected with marrow cells of the low density region (21 to 25 percent BSA) had spleens positive for anti-burro PFC when immunized with BRBC, but negative for anti-sheep PFC when immunized with SRBC. Sixty and 56 percent of mice injected with a pool of cells from all fractions (*10*) had spleens positive for anti-sheep or anti-burro PFC, respectively. Considering that about 10 percent of all marrow cells subjected to centrifugation were recovered from the low density fractions (*4*), enrichment with precursor cells of anti-burro PFC must have occurred. However, most of such precursors were not capable of generating anti-sheep PFC upon interaction with thymocytes and SRBC. Presumably, the precursors concentrated in the fractions of 21 to 25 percent BSA were restricted for the specificity of antibody. The lack of anti-sheep responses indicated that precursors of anti-sheep PFC were depleted in the low density fractions.

A great proportion of recipients of the 31 percent BSA fraction had spleens positive for anti-sheep PFC upon immunization with SRBC, but not for anti-burro PFC upon injection of BRBC. Since the yield of nucleated cells in this fraction was about 30 percent of input (*4*), enrichment of precursor units for anti-sheep PFC could have been marginal, but the depletion of anti-burro PFC precursors must have been substantial. This pattern was the opposite of that seen in the lower density region and, likewise, was compatible with the hypothesis that precursors of PFC were restricted for antibody specificity. Most recipients of the intermediate density fractions (27 to 29 percent BSA) had spleens positive for either anti-sheep or anti-burro PFC. These fractions may have contained the two types of restricted marrow cells or nonrestricted precursors of PFC, or both. In summary, precursor cells of two different specificities were depleted or concentrated in various regions of the gradient. Depletion and enrichment of the two kinds of cells were unequally distributed along the gradient.

Most spleens that were positive for direct PFC were also positive for indirect PFC of the same specificity, and vice versa. The probability that a common marrow precursor cell generated PFC of the IgM and IgG classes was > .995 for anti-sheep and anti-burro responses, as determined by chi-square tests of independence (Table 1). This was in full agreement with results of limiting dilution assays for marrow pre-

Table 2. Frequencies of antibody responses (direct PFC) to SRBC and BRBC in irradiated recipients of fractionated marrow cells, 4×10^7 thymocytes, and 5×10^8 xenogeneic erythrocytes; +, recipient spleen with > 200 PFC; G.M., geometric mean PFC/positive spleens; −, not done.

Fraction (% BSA)	Anti-SRBC + spleens/ No. grafted	Anti-SRBC G.M.	Anti-BRBC + spleens/ No. grafted	Anti-BRBC G.M.
	1.25×10^5 marrow cells			
23–25	−		9/12	399
27–29	10/12	380	9/9	489
31	7/8	388	−	
	5×10^5 marrow cells			
23–25	2/17	273	−	
31	−		1/5	575

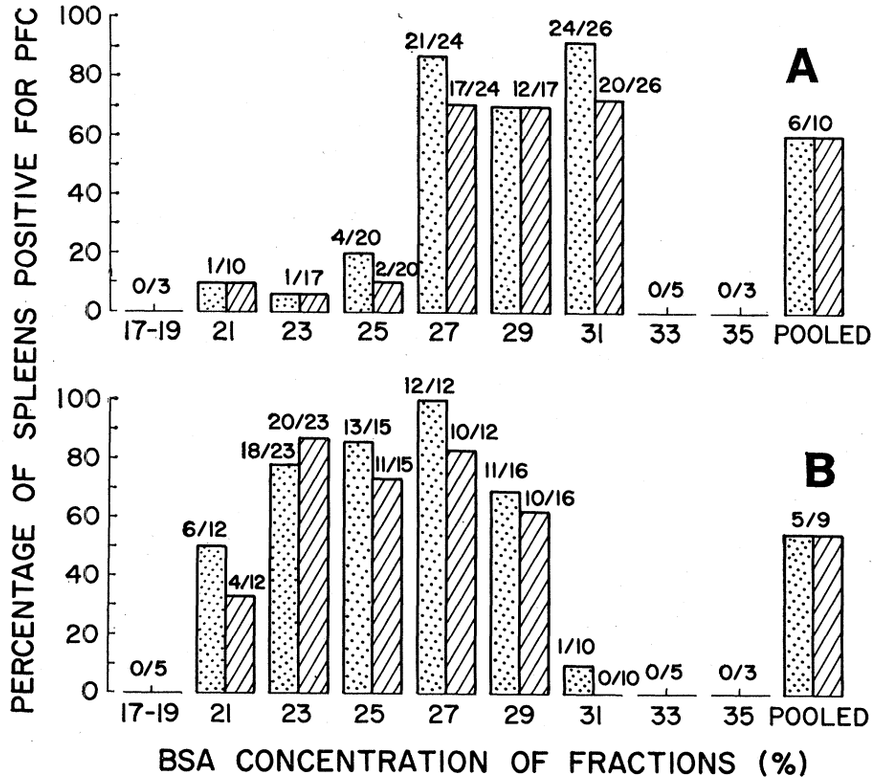

Fig. 1. Numbers and percentages of spleens positive for direct (stippled bars) and indirect (striped bars) plaque-forming cells 9 days after irradiation of mice and transplantation of 4×10^7 syngeneic thymocytes with 2.5×10^5 fractionated or pooled (far right) marrow cells that were subjected to equilibrium density gradient centrifugation. Immunization of cell-injected mice was either with sheep (A) or burro (B) erythrocytes, and the assays for plaque-forming cells were against erythrocytes of the species used for immunization.

cursors of anti-sheep PFC in (C57BL/6 × DBA/2)F_1 mice, but differed from results obtained with marrow cells of other strains (*3, 4*). Class differentiation of potentially immunocompetent marrow cells is apparently controlled by genetic factors that are not determinant-specific. Another genetic control of this type was described for mouse serum levels of immunoglobulins and of agglutinins to sheep and pigeon erythrocytes (*11*).

It was desirable to subject the results obtained by transplanting fractionated marrow cells (2.5×10^5 cells per mouse) to a more rigorous test. For this reason, twice as many cells of the fractions with 23 and 25 percent BSA were tested for anti-sheep PFC, and half as many cells for anti-burro PFC (Table 2). This change in the numbers of grafted marrow cells did not obscure the depletion of precursors of sheep specificity nor the enrichment of precursors of burro specificity. Results of similar variations of the numbers of transplanted marrow cells from the 31 percent BSA fraction strengthened the conclusion that in this region of the gradient precursors of anti-burro PFC were depleted instead of precursors of anti-sheep PFC.

The data are best explained by specificity differentiation of marrow cells responsible for PFC production, presumably of the precursor cells of PFC. Restriction for antibody specificity preceded that for immunoglobulin class in this mouse strain. Although differing density gradient profiles of specific splenic precursors of antibody-forming cells were resported (*12*), these data are the first direct demonstration of commitment to antibody specificity in less differentiated and mature cells of the immune system. The separation of restricted marrow cells could have been due to osmotic rather than to density gradients (*13*), but in either case it was not clear why physical parameters of precursors for SRBC and BRBC should differ. It is possible that prior exposure of the cells to immunogens cross-reacting with one or the other of the erythrocytes caused commitment. However, it would be unrealistic to expect density differences such as those reported here between marrow precursors for all specificities.

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8 March 1971

Transcription of Nonrepeated DNA in Mouse Brain

Abstract. *Under normal conditions of DNA renaturation, about 60 percent of mouse DNA fragments renature at a rate consistent with their being present only once per sperm. These nonrepeated sequences (also called single-copy or unique) may be used in RNA-DNA hybridization experiments to provide quantitative estimates of RNA diversity. About 10 percent of the mouse single-copy sequences are transcribed in mouse brain tissue. Estimates of about 3 percent were obtained for mouse liver and kidney RNA's. If only one of the complementary DNA strands is transcribed, this hybridization value implies that the equivalent of at least 300,000 different sequences of 1000 nucleotides are expressed in mouse brain tissue. It is suggested that the large amount of DNA in mammals is functionally important, and that a substantial proportion of the genome is expressed in the brain.*

Estimates on the extent of transcription of RNA from DNA in various eukaryotic cells are usually based on experiments in which RNA is hybridized to denatured DNA immobilized in agar or on nitrocellulose filters (*1*). These data are interpreted in a qualitative manner because of experimental limitations imposed by most eukaryotic DNA's. Renaturation kinetics of single-strand, sheared DNA's are consistent with the suggestion that many eukaryotic genomes contain repeated (rapidly renaturing) and nonrepeated (also termed single-copy or unique) nucleotide sequences (*2*). In view of the low RNA concentrations and the relatively short time allowed for reaction, it is likely that many hybridization estimates of transcriptional diversity pertain to the repeated fraction of the eukaryotic genome. This latter inference is supported by physical characterization of such RNA-DNA hybrids. Thermal stability measurements indicate that incomplete base pairing occurs in contrast to the much more precise base pairing expected for RNA hybridized to nonrepeated DNA. Thus, while important conclusions can be drawn from this type of RNA-DNA hybridization, the quantitative aspects of transcription in eukaryotic cells are not well understood. This information is required for a better understanding of the control of cell and tissue differentiation, and for insight into the functional significance of the enormous sequence complexity of most eukaryotic genomes (*2, 3*).

Methods of assaying transcription of nonrepeated DNA have been developed and applied to *Xenopus* oocytes and fetal mice (*4*). We have examined the transcriptional representation of the nonrepeated DNA fraction in RNA's from mouse liver, kidney, and brain. Our results indicate that RNA from the brain hybridizes to a large fraction of the nonrepeated mouse DNA. The RNA's from mouse liver and kidney also represent extensive diversity of nucleotide sequence, although hybridization values for RNA's from these tissues are about one-third those obtained for brain RNA's. These data are in accord with the results of Smith (*5*) obtained by hybridizing labeled RNA to filter-bound, unique, mouse DNA.

For our experiments we used radio-