

## IMMUNOLOGICAL MEMORY IN MICE

### I. PHYSICAL SEPARATION AND PARTIAL CHARACTERIZATION OF MEMORY CELLS FOR DIFFERENT IMMUNOGLOBULIN CLASSES FROM EACH OTHER AND FROM ANTIBODY-PRODUCING CELLS\*

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The injection of sheep red blood cells into mice leads to the proliferation of antigen reactive cells, the production of specific antibodies, and the development of an enlarged and/or altered population of antigen reactive cells (1). These events provide the animal with an immunological memory, so that when restimulated with the same antigen, antibodies will be produced more rapidly and in large quantities. By extension from other antigenic systems (2), these antibody molecules may have higher affinity.

Although it is well established now that each mature antibody-producing cell makes only one species of antibody which is homogeneous with respect to specificity, H chain class, L chain type, and allotype (3-7), it is neither established whether these antibody-producing cells derive from a common or separate cell lines nor whether memory cells are committed as to class or allotype. Finally, the relationship of memory cells to antibody-producing cells is also not yet clear. Are memory cells antibody-producing cells, or derived from them?

Answers to these questions can be obtained by separating the various classes of antibody producing cells and memory cells and by establishing the kinetics of their appearance relative to one another. In this paper we used the procedures of bovine serum albumin (BSA) density gradient centrifugation (8, 9) to separate spleen cells from animals immunized with sheep red blood cells (SRBC), and analyzed the fractions obtained for their content of plaque-forming cells (PFC) by the localized hemolysis in gel method of Jerne et al. (10). The memory cell activity of the fractions was estimated in an adoptive transfer system. In some experiments, PFC were completely depleted from cell suspensions by adsorption onto glass bead columns (11) to determine what effect this had on the ability of these cell suspensions to adoptively transfer the immune response of the several classes. The kinetics of density changes in PFC and

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memory cells were examined as a means of establishing the time of development of the different classes of these two kinds of cells.

### *Materials and Methods*

*Mice.*—The congenic strains used in these experiments are essentially histocompatible and genetically identical at all loci except those in the Ig chromosome region and a small adjacent region on that chromosome (12, 13). Strains CWB/5 and CWB/8 (Ig<sup>b</sup>)<sup>1</sup> were obtained by crossing C57BL/10 (Ig<sup>b</sup>) to C3H.SW SnHz (CSW) (Ig<sup>a</sup>) and by back-crossing the F<sub>1</sub> hybrid to CSW five and eight times respectively, always using an Ig<sup>b</sup> positive animal of the previous back-cross generation. These strains have since been maintained by brother by sister mating of Ig<sup>b</sup> homozygotes. Intercurrent selection for skin compatibility of animals to be back-crossed was made in back-cross generations two and three, resulting in complete skin compatibility and a high degree of congenicity by the fifth back-cross generation (13). In order to conform to the practice recommended by the International Committee on Nomenclature for Inbred Strains of Mice (14), we are continuing to back-cross the CWB strain to the 14th generation, but since no differences were obtained in the experiments here reported using CWB/5 and 8, the results are reported together. Within a single transfer experiment donor and host mice were always of the same sex, but of different Ig allotypes. Mice to be used as lymphoid cell donors were between 6 and 9 wk of age at the time of immunization. Recipients were between 10 and 16 wk of age at the time of cell transfer.

*Irradiation.*—4–6 hr prior to cell transfer, all recipient mice received 600 rads total body X-irradiation using a Siemens X-ray machine operating under the following conditions: 250 kv, 15 ma, 0.25 mm. Cu and 1.0 mm AL, and a HVL (half-value layer) of 1.10 mm Cu. The mice were contained in a circular perforated plastic box with an individual compartment for each mouse. The dose rate was 80 rads/min, and the focal skin distance was 60 cm.

*Immunization.*—Mice were given a single intravenous injection of  $4 \times 10^8$  sheep erythrocytes (SRBC) suspended in 0.1 ml of (MEM-PM).<sup>2</sup> In some experiments  $4 \times 10^5$  SRBC were used. The SRBC obtained in Alsever's solution were used 2–4 wk after bleeding and were washed three times in normal saline prior to use.

*Spleen Cell Suspensions.*—Mice were killed by cervical dislocation, their spleens were removed, and a single cell suspension was obtained by gently pressing the tissue in a few drops of MEM-PM through a 50-mesh stainless steel screen. Cell clumps were broken up by repeated aspiration through a pipette, and the suspension was allowed to stand for 2–3 min after which the supernatant was carefully removed and centrifuged at 300 g for 10 min. The cells were resuspended in MEM-PM and maintained at melting ice temperature throughout the experiments. Cell counts were obtained using a Model B Coulter Counter fitted with a 100  $\mu$  aperture and using optimum window setting previously calibrated with hemocytometer counts of nucleated spleen cells.

*Assay for Plaque Forming Cells (PFC).*—The numbers of direct ( $\gamma$ M or 19S) and indirect (developed or  $\gamma$ G) PFC in spleen cell suspensions were determined using a modification of

<sup>1</sup> The designations Ig<sup>a</sup> and Ig<sup>b</sup> without specification of locus mean that all the Ig loci on that chromosome have the respective a or b alleles. Since the Ig loci are so closely linked that recombinants have not been observed in inbred strain matings, this is a useful shorthand.

<sup>2</sup> MEM-PM is minimum essential medium (Eagle) Cat. No. F-12 instant tissue culture powder medium without NaHCO<sub>3</sub> (Grand Island Biological Co., Oakland, Calif.) made up with Na<sub>2</sub>HPO<sub>4</sub>·12H<sub>2</sub>O (358 mg/liter) and MgCl<sub>2</sub>·6H<sub>2</sub>O (200 mg/liter) instead of bicarbonate, in distilled, deionized water.

the hemolysis in gel method originally described by Jerne et al. (10), adapted for microscope slides by Mishell and Dutton (15), and extended for development of indirect or  $\gamma$ G PFC by Dresser and Wortis, Sterzl and Riha, and Weiler et al. (16-18). Portions of 0.25 ml of Agarose, 0.5% in MEM-PM, were maintained at 47°C in 10 × 75 mm tubes. SRBC (15  $\mu$ l of 20% v/v) and 10-20  $\mu$ l of lymphoid cell suspension were added to each tube just prior to spreading on glass slides that had previously been coated with 0.1% Agarose and allowed to dry. The slides were inverted in recessed racks, which allowed the application of constant amounts of incubation media. Slides were first incubated for 1 hr at 37°C with MEM-PM, and then were transferred to clean racks. Three sets of slides in duplicate were used. To assay for direct PFC, one set of slides was then incubated with normal BALB/C mouse serum<sup>3</sup> at the dilution of 1:100 to 1:200 in MEM-PM to correspond with the concentration of mouse anti-allotype sera used to develop  $\gamma$ G<sub>2a</sub> PFC. For detection of total  $\gamma$ G PFC, a second set of slides was exposed to a polyvalent rabbit anti-mouse  $\gamma$ G (serum) at a dilution of 1:1000. The third set was developed with anti-allotype sera to reveal  $\gamma$ G<sub>2a</sub> PFC. After this second incubation of 1 hr at 37°C, all slides were transferred to clean racks and incubated for an additional hour in the presence of complement<sup>4</sup> diluted 1:10 with MEM-PM. All sera and complement used in those experiments had previously been absorbed with equal volumes of packed SRBC in the cold. The number of  $\gamma$ G and  $\gamma$ G<sub>2a</sub> PFC was determined by subtracting the number of direct PFC from the total number of PFC developed with the antisera.

*Antisera for Development of Indirect PFC.*—Polyvalent rabbit anti-mouse  $\gamma$ G is a large pool (pool A) of sera obtained by immunization with  $\gamma$ G of several mouse strains. It precipitates in high titers <sup>125</sup>I-labeled proteins of all classes of mouse  $\gamma$ G and Fc fragments of these classes and has a lower, but definite, titer against Fab. It is difficult to make a direct comparison between these titers and efficiency of development and/or inhibition of PFC (19). Pool A was used at a dilution of 1:1000, the dilution previously determined to give maximum numbers of developed PFC with spleen cells obtained from mice 7-10 days after immunization. The mouse anti-allotype sera used for development of  $\gamma$ G<sub>2a</sub> plaques were produced in mice immunized with *Bacillus proteus* anti-*proteus* aggregates or antisera directed against the H-2 antigens of the anti-allotype sera producers (20). For "a" PFC development, anti-allotype serum pools made in C57BL mice against CSW anti-*proteus*-*B. proteus* aggregates were used. Such pools are potentially reactive against the four classes  $\gamma$ G<sub>2a</sub>,  $\gamma$ A,  $\gamma$ G<sub>2b</sub>, and  $\gamma$ G<sub>1</sub> corresponding respectively to the loci Ig-1, 2, 3, and 4. However, pools selected for use in these studies had detectable precipitation only with <sup>125</sup>I-labeled  $\gamma$ G<sub>2a</sub> myeloma proteins (of the "a" allotype). Therefore, development with these sera at their limiting dilutions revealed only  $\gamma$ G<sub>2a</sub> PFC.

For the development of "b" PFC two pools of anti-allotype serum were used. These sera did not precipitate a  $\gamma$ G<sub>1</sub> myeloma protein of "b" allotype (MOPC300, kindly supplied by Dr. M. Potter), although it did precipitate in high titer a  $\gamma$ G<sub>2a</sub> protein preparation obtained from a SJL/J mouse with a hypergammaglobulinemia of this class (21). Since myeloma protein of all classes of "b" allotype are not available the sera could not be tested for reactivity with  $\gamma$ G<sub>2b</sub>. Therefore, for "b" PFC development we can say with certainty only that  $\gamma$ G<sub>2a</sub> PFC are revealed and that  $\gamma$ G<sub>1</sub> are not. The dilution of anti-allotype sera used were limiting dilutions (usually between 1:100 and 1:200) previously found to develop the maximum number of  $\gamma$ G<sub>2</sub> PFC in spleens removed from mice 8-10 days after immunization.

*Separation of Lymphoid Cells.*—Separation of cells was achieved by isopycnic (equilibrium) centrifugation in a stepwise density gradient of BSA using the method described by Raidt

<sup>3</sup> Normal BALB/C mouse serum was found to increase the visibility of direct plaques and gave 10-30% higher direct PFC counts than slides incubated without serum addition.

<sup>4</sup> Dried complement (guinea pig) Hyland Laboratories, Los Angeles, Calif.

et al. (9). As reported by these authors, results vary with the lot of BSA solution used. In these experiments a single lot<sup>5</sup> of 35% sterile isotonic solution of BSA, pH 7 and specific conductance 5.2 millimhos was used. 1 ml each of 33%, 29%, 23%, and 0.5 ml of 10% BSA diluted in balanced saline solution (BSS) (9) was layered in a nitrocellulose tube. The cells to be separated were included in the 33% layer. The tubes were spun in the SW39 Beckman rotor at 13,500 rpm for 30 min at 4°C. Four fractions, A, B, C, and D were harvested from the interphases commencing at the top. The harvested fractions were suspended in 20 volumes of MEM-PM, centrifuged at 300 g for 10 min at 4°C, and resuspended in MEM-PM.

*Fractionation of Spleen Cells on Glass Bead Columns.*—The method described by Plotz and Talal (11) was used to further separate density fractions into adherent and nonadherent populations. Glass beads<sup>6</sup> in MEM-PM containing 10% fetal calf serum were packed in a 5 ml plastic syringe, occupying a volume of approximately 3 ml. This was equilibrated at 37°C, and 70–90 million nucleated cells were loaded onto each column. After 10 min, non-retained cells were eluted by washing with about 30 ml of the same medium at 37°C. Some adherent cells were eluted with 1% ethylenediaminetetraacetic acid (EDTA) in cold Puck's Saline G (22) without divalent cations. Recovery in these two fractions is reported in Results.

*Experimental Design.*—The general plan of the experiments is indicated in Fig. 1. Spleens or lymph nodes were removed from mice 3–118 days after intravenous injection of  $4 \times 10^8$  SRBC (in some experiments  $4 \times 10^5$ ) and a single cell suspension was prepared. The cells, pooled from four to eight spleens, were separated according to density on the BSA gradient into fractions, A, B, C, and D. In some experiments gradient fraction A was further separated on glass bead columns.

Aliquots of the original cell suspension and of all fractions obtained after separation procedures were assayed for their PFC content. Equal numbers of nucleated cells from each cell suspension were injected into mice irradiated with 600 R together with  $4 \times 10^8$  SRBC. 7 days later, the numbers of PFC in the spleens of these recipient mice were determined.

## RESULTS

*Specificity and Extent of PFC Development by Anti-Allotype Sera.*—The specificity of development with anti-allotype sera is demonstrated in Table I. Anti-“a” sera were able to develop plaques ( $\gamma G_{2a}$  PFC as described in Materials and Methods) only when the spleen cells were taken from immunized CSW mice or from CWB mice that had been irradiated and given CSW cells of immunized donors. Similarly anti-“b” sera developed plaques only with spleen cells taken from CWB mice or from irradiated CSW mice given primed CWB cells. As described in Methods the “b” developed PFC include  $\gamma G_{2a}$  producing cells and do not include  $\gamma G_1$  producing cells. Gamma  $G_{2b}$  PFC possibly are developed with anti-“b” sera (see Materials and Methods).

*Parameters for Demonstration of an Adoptive Secondary Response (“Memory”).*—Preliminary experiments were carried out to determine the optimum time between cell transfer and plaque assay giving maximum numbers of direct and developed PFC. This was found to be 7 days using cells taken from animals 3–10 days after primary immunization—in reasonable agreement with the re-

<sup>5</sup> Pentex, Inc., Kankakee, Ill., Path-o-cyte No. 5, Lot 10-2.

<sup>6</sup> Superbrite glass bead, type 150-5005, Minnesota Mining & Manufacturing Co., Reflective Products Div., St. Paul, Minn.

ports of others (23-25). In most of our studies a primary saturating immunizing dose of  $4 \times 10^8$  SRBC was used. A low priming dose of  $4 \times 10^5$  SRBC resulted in a very low primary PFC response but in a higher secondary response after transfer than was obtained when  $4 \times 10^8$  SRBC were used for priming (26). To

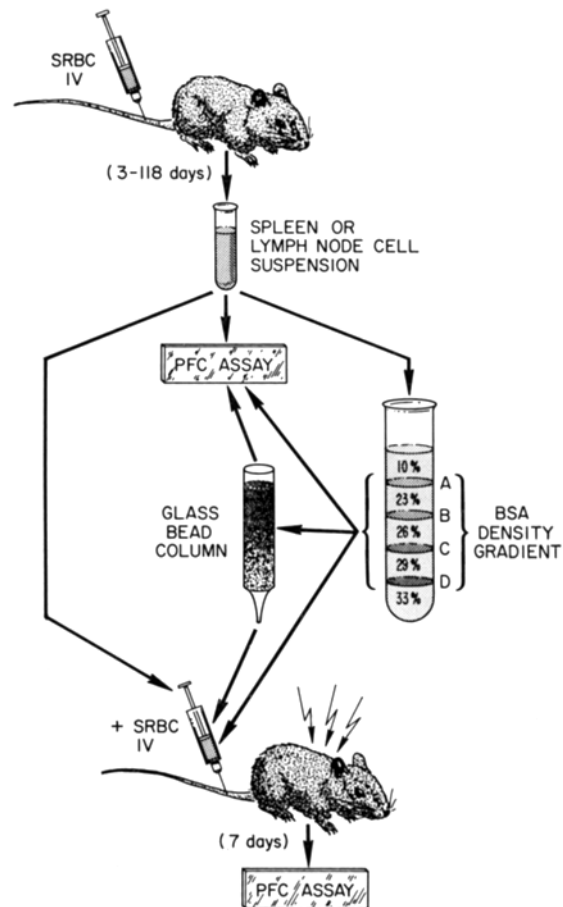


FIG. 1. Experimental design.

obtain the secondary adoptive response a challenge dose of  $4 \times 10^8$  SRBC was injected along with the spleen cells in all experiments.

Various numbers of spleen cells from normal and immunized mice were transferred into irradiated recipients to determine the dose that would consistently result in an adoptive response of developed PFC with primed cells but would not give significant numbers of developed PFC with unprimed cells. More than

$5 \times 10^6$  cells from nonimmunized mice were needed to elicit a significant response, while detectable numbers of PFC were obtained using as few as  $5 \times 10^5$  cells from primed animals. Consistently high adoptive secondary responses for all  $\gamma G$  classes of developed PFC were obtained with  $5 \times 10^6$  transferred cells, and this dose was used for most of the experiments.

TABLE I  
*Demonstration of Specificity of Development of  $\gamma G_{2a}$  PFC in Spleen Cell Suspensions with Anti-allotype Sera*

Source of spleen cells*	Ig-1 allotypes	No. of PFC on duplicate slides†		
		Direct	Developed with:	
			Anti-a	Anti-b
Immunized CSW	a	70,60	130,118	58,68
		42,30	103,101	33,35
		66,58	160,157	61,64
Immunized CWB	b	22,29	20,27	93,81
		40,41	38,43	85,77
		124,143	140,130	212,230
Irradiated CSW given spleen cells from immunized CWB mice	b → a	8,12	9,12	45,55
		59,73	71,61	112,115
		82,74	75,69	232,210
Irradiated CWB given spleen cells from immunized CSW mice	a → b	29,30	52,58	32,30
		80,86	124,138	81,77
		64,62	145,127	63,58
Irradiated CSW given spleen cells from normal CWB mice	b → a	52,44	50,56	49,44
		87,91	70,100	83,100
		101,105	105,103	108,92

\* Spleen cell pools from four to eight donor mice.

† PFC assays performed 7 days after SRBC injection into normal mice or after spleen cell transfer ( $5 \times 10^6$  cells) and simultaneous injection of SRBC into irradiated mice. The figures in the various lines indicate values obtained in different experiments in which from  $0.2$  to  $2.2 \times 10^6$  cells were plated.

*Distribution of Direct and Indirect PFC in BSA Gradient Fractions.*—Pooled spleen cells taken from 4–8 mice on day 7–10 after immunization were fractionated by density gradient centrifugation into four bands (A, B, C, D). Total cell recovery varied from 55–70%. The remainder of the cells was lost presumably in the pellet or during the washing procedures. Of the cells recovered in the four bands in all experiments 15–23% were found in A, 34–43% in B, 28–30% in C, and 11–14% in D. The means of the numbers for direct, total  $\gamma G$  and  $\gamma G_{2a}$  PFC

per  $10^6$  nucleated cells in the four bands, expressed relative to the numbers found in the unfractionated spleen cell suspension and calculated separately for each experiment, are indicated in Fig. 2. The average number of direct PFC per  $10^6$  cells was significantly increased in bands A and B, unchanged in band C, and significantly decreased in band D. Similar results have been reported for

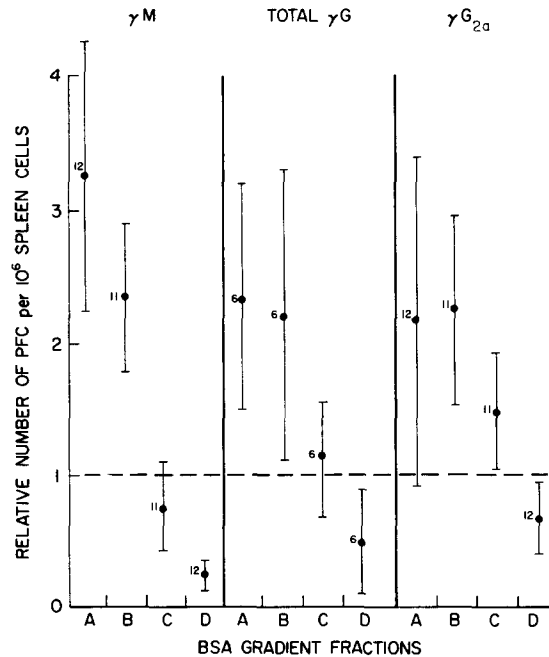


FIG. 2. Relative distribution of the three classes of PFC in BSA gradient fractions of spleen cells taken from mice 7–10 days after immunization. The points represent the arithmetic mean of the values for PFC/ $10^6$  cells in four gradient bands relative to the value obtained for unseparated spleen cell suspensions (horizontal dashed line). The numbers on the points indicate the numbers of experiments (four to eight spleens pooled per experiment). The vertical bars represent the 95% confidence limits computed by using appropriate Student *t*-test values.

splenic direct PFC 1–3 days after immunization (9). An enrichment of total indirect PFC was obtained in bands A and B and a depletion in band D. The  $\gamma G_{2a}$  PFC were more equally distributed between the three upper bands. Only the D-band showed significantly decreased numbers.

*Distribution of Memory Cells in BSA Gradient Fractions.*—Splenic memory cells from animals 7–10 days after immunization showed similar distributions in BSA gradients and the numbers of direct and developed PFC obtained in the recipient spleens 7 days after transfer of  $5 \times 10^6$  cells from each gradient frac-

tion are reported in Figs. 3 and 4. The points in Fig. 3 represent the geometric means of the values for PFC per recipient spleen obtained in all experiments done for each group. The points in Fig. 4 represent the geometric mean of normalized differences for each individual experiment between unseparated spleen cells and cells from the gradient fractions. Unseparated spleen cells and cells

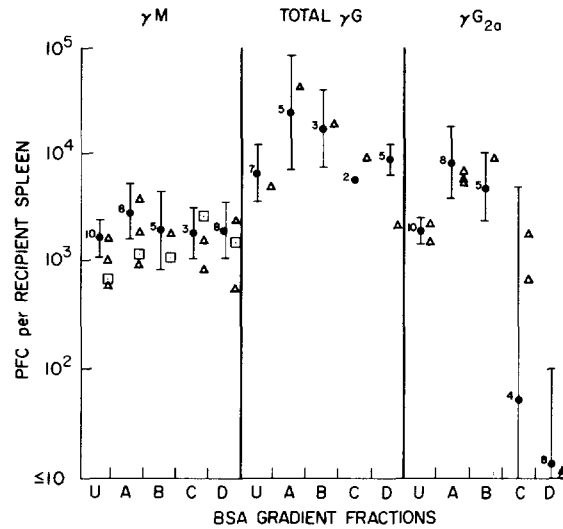


FIG. 3. Distribution in BSA gradient fractions of memory for  $\gamma M$ , total  $\gamma G$ , and  $\gamma G_{2a}$  PFC and ability to transfer a primary adoptive response for  $\gamma M$ . Spleen cells from donor mice were separated 7–10 days after primary immunization, and  $5 \times 10^6$  cells from each fraction were transferred together with  $4 \times 10^8$  SRBC into irradiated recipients. U, unseparated spleen cells; A, B, C, D, gradient fractions. Solid circles (●) represent the geometric mean for the numbers of PFC per recipient spleen (antilog of average of  $\log [x + 1]$  where  $x$  = number of PFC). The vertical bars represent the 95% confidence limits. Numbers next to the points represent numbers of experiments (pool of two to three recipient spleens per experiment).  $\Delta$  represents values obtained with lymph node cells (pool of two recipient mice per point).  $\square$  represents values obtained with spleen cells from unimmunized donors (points are geometric means of three experiments with two recipient spleens pooled for each experiment).

from the four bands were about equal in their ability to give rise to direct PFC in the irradiated recipients. Fraction A was significantly enriched (Fig. 4) for total  $\gamma G$  memory while the other fractions were not significantly different (at the 5% level) from the unseparated spleen cells in their ability to give rise to total  $\gamma G$  PFC. However, for  $\gamma G_{2a}$  a marked difference was found following injection of equal numbers of cells from the various bands. There is a four-fold enrichment for  $\gamma G_{2a}$  PFC memory in the top (A-band) and then a progressive decrease in B and C with finally a very considerable depletion for  $\gamma G_{2a}$  memory



in the D-band. The D-band, 7–8 days after immunization, had no detectable  $\gamma G_{2a}$  memory (see also Fig. 5). Thus, D-band cells, though unchanged in capacity for direct and total indirect PFC memory, were virtually unable to transfer a  $\gamma G_{2a}$  PFC response.

In a few experiments, similar results for memory distribution of the various

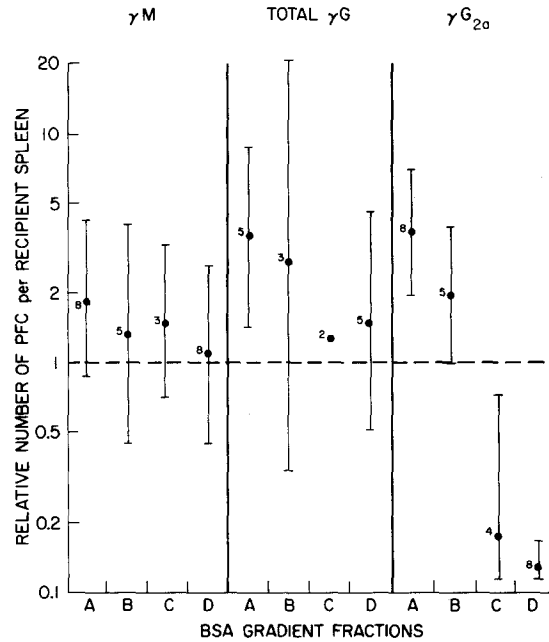


FIG. 4. Relative distribution of memory for three classes of PFC in BSA gradient fractions. Irradiated recipients were injected intravenously with  $5 \times 10^6$  cells from each gradient fraction or from unfractionated spleen together with  $4 \times 10^8$  SRBC. The points represent the geometric means of the numbers of PFC obtained 7 days later in spleens of recipients injected with cells from BSA gradient fractions relative to unseparated spleen cells (dashed horizontal line). Numbers next to points represent numbers of experiments (two to three recipients per experiment). Vertical bars indicate 95% confidence limits.

classes were found with spleen cells separated 4–7 days after immunization with  $4 \times 10^5$  SRBC.

Though lymph nodes of mice immunized intravenously with  $4 \times 10^8$  SRBC showed little or no primary PFC response, the adoptive response with lymph node cells 8 and 9 days after immunization paralleled that with spleen cells. The gradient distribution for memory was similar to that of spleen cells (triangles in Fig. 3). Also included in Fig. 3 are results of adoptive transfer of unimmunized spleen cells. The mean numbers of direct ( $\gamma M$ ) PFC obtained after transfer of  $5 \times 10^6$  unimmunized spleen cells from the various gradient fractions did not

significantly differ from the results obtained with immunized cells. Nevertheless, no developed PFC ( $\gamma$ G) were obtained after transfer of cells from unimmunized donors.

*Change in Density with Time after Primary Immunization of  $\gamma$ G<sub>2a</sub> Memory Activity.*—Groups of mice were killed 3–118 days after an injection of  $4 \times 10^8$  SRBC, and A- and D-band cells prepared from spleen and lymph nodes were injected into irradiated recipients. As shown in Fig. 5, D-band cells at

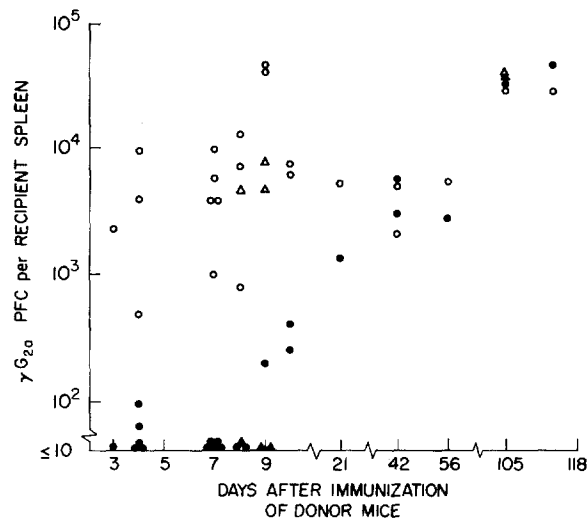


FIG. 5. Change in BSA gradient distribution of memory for  $\gamma$ G<sub>2a</sub> PFC with time after immunization. Separation of donor spleens on BSA density gradients was done 3–118 days after primary immunization with  $4 \times 10^8$  SRBC.  $5 \times 10^6$  separated cells of A- or D-band were injected with  $4 \times 10^8$  SRBC into irradiated recipients. Each point represents  $\gamma$ G<sub>2a</sub> PFC obtained in one to three recipient spleens 7 days after transfer of A-band cells (○, spleen; △, lymph nodes) or D-band cells (●, spleen; ▲, lymph nodes).

3–9 days after priming displayed no or a markedly reduced capacity to transfer  $\gamma$ G<sub>2a</sub> memory when compared with an equal number of A-band cells. From 10 days the A to D difference decreased and no difference between A- and D-band cells was evident by 42 days and up to 118 days after immunization.

*Absence of Correlation between Numbers of PFC and Memory Activity in BSA Gradient and Glass Bead Column Fractions.*—The results presented in Fig. 2 (distribution of PFC in the gradient fractions) and in Figs. 3 and 4 (distribution of memory cells) indicated that there is only a partial parallelism between the numbers of PFC in any one band and the capacity to engage in an adoptive secondary response. Thus the D-band contains a lower number of PFC of all types, yet only the adoptive  $\gamma$ G<sub>2a</sub> PFC response is deficient. The A-band is en-

riched for all PFC types, but contains a significantly increased number of memory cells only of  $\gamma G_{2a}$  type. The following experiments were designed to determine whether removal of all PFC types from the A-band would result in a decreased capacity to transfer memory. Using a glass bead column the cells were separated into two fractions: one containing cells that were nonadherent to glass at 37°C ("nonretained") and a second containing cells that were adherent at 37°C in MEM-PM but were eluted in the cold with medium free of divalent cations and containing EDTA ("retained and eluted"). These fractions

TABLE II  
*Separation of PFC from Cells Capable of Transferring an Adoptive Immune Response Using Glass Bead Columns*

Cells	PFC per 10 <sup>6</sup> cells before injection ( $\times 10^{-2}$ )			% Cell recovery	Total PFC/recipient spleen per 10 <sup>6</sup> injected nucleated cells ( $\times 10^{-2}$ )		
	Direct	Indirect			Direct	Indirect	
		$\gamma G_{2a}$	$\gamma G$ total			$\gamma G_{2a}$	$\gamma G$ total
Fraction "A" (from BSA gradient)	2.7* (1.3-5.9)	2.5 (2.3-2.7)	11.5 (9.1-15.8)		23‡ (16-33)	26 (23-30)	111 (79-133)
Nonretained	0.02 (0-0.05)	0.03 (0.01-0.04)	0.2 (0-0.4)	14.2* (6.5-18.8)	26 (19-54)	58 (47-72)	273 (168-400)
Retained and eluted	1.3 (0.3-2.5)	1.5 (1.0-2.1)	6.7 (4.3-8.7)	35.2 (30.5-42.2)	15 (2-73)	11 (3-44)	113 (26-475)

\* Arithmetic means (range).

‡ Geometric means (range).

The data are from three experiments. For each experiment, spleen cells of six to seven donor mice 7 days after immunization with  $4 \times 10^8$  SRBC were pooled and separated on a BSA gradient. Fraction A was then separated into a nonretained and a retained and eluted fraction (see Methods) on a glass bead column. Two to three irradiated mice received  $10^6$  cells plus  $4 \times 10^8$  SRBC each, and their spleens were individually assayed 7 days later for PFC.

contained respectively about 14% and 35% of the A-band cells, leaving about 50% still adherent to the glass beads. PFC assays of these fractions were performed and, as shown in Table II, the nonretained cell fraction was virtually devoid of direct, total indirect, and  $\gamma G_{2a}$  PFC. However, one million cells from this fraction were able to elicit in irradiated recipients a memory response for all PFC types, which was in point of fact higher than that of unfractionated A-band cells. It is also clear from Table II that the fraction eluted from the column contained numerous PFC and that these cells were as effective in transferring a secondary PFC response as unseparated A-band cells.

#### DISCUSSION

Sheep erythrocytes stimulate sensitive cells to undergo metabolic changes resulting in proliferation and the subsequent formation of antibody producing

cells. Recently, substantial evidence has revealed that this involves the interaction of two cells derived respectively from the thymus and bone marrow in the mouse (27, 28). Although both may be sensitive to antigen, the latter is the major source of precursors of the actual antibody producing cells (29) detected by the localized hemolysis in gel techniques. We have utilized the buoyant density changes that occur in stimulated cells to show the relationship between the PFC of different immunoglobulin classes and the cells conferring immunological memory.

This report is the first to our knowledge of the distribution in density gradients of total  $\gamma$ G and  $\gamma$ G<sub>2a</sub> PFC, although studies of others have shown that  $\gamma$ M PFC have a lower average density in BSA density gradients than the bulk of nucleated spleen cells (9, 30). During the period of 7–10 days after antigenic stimulus, total  $\gamma$ G PFC, mainly of the  $\gamma$ G<sub>1</sub> type, are also enriched in the less dense fractions but not as much as the  $\gamma$ M PFC.  $\gamma$ G<sub>2a</sub> plaques have a slightly flatter distribution across the density gradient. These differences in the density profile between  $\gamma$ M and  $\gamma$ G producing cells at that stage of immunization undoubtedly reflect differences in the physiological state of these cells. Cells entering into a metabolically active state such as occurs on blast transformation are lighter than the quiescent lymphocytes from which they arise. As these cells approach division they can be expected to become still less dense. Antigen sensitive cells 10 hr after stimulation are much lighter than prior to antigenic stimulation (31) but plasma cells are, on the average, denser than either 19S PFC or total spleen cells (30). Erythrocytes become progressively more dense as they age (32).

The lower density of  $\gamma$ M PFC therefore is consistent with their recent derivation from dividing cells. In fact, PFC of the  $\gamma$ M type have themselves been seen to divide (33) and Jerne et al. (10) have suggested that  $\gamma$ M PFC have half-lives of less than 24 hr. Studies utilizing thymidine incorporation have shown that all 19S PFC have recently synthesized DNA (34, 35). The appearance of relatively fewer  $\gamma$ G<sub>2a</sub> PFC in the lighter fractions and enrichment in the denser fractions at 7–10 days after immunization may be a consequence of the longer time after division required for a cell to secrete  $\gamma$ G<sub>2a</sub> antibodies in sufficient quantities to become a PFC.

Antigenic stimulation causes not only a density change of cells producing antibodies, but also of cells capable of transferring a secondary response, i.e. memory. The density profile of memory cells for the different classes is quite distinct from that of the PFC of these classes. Our aim in this work was to study the  $\gamma$ G PFC and memory response, but our data also bear on  $\gamma$ M memory. We find that there is neither an appreciable density change subsequent to 7 days after an immunizing dose of  $4 \times 10^8$  SRBC in cells capable of transferring a  $\gamma$ M response, nor is there an increase in the response transferred by  $5 \times 10^6$  cells from immunized compared with nonimmunized animals. Thus, if there is

$\gamma$ M memory under these conditions, it persists for less than 7 days. Cunningham (25) and Shearer et al. (24) have recently looked for  $\gamma$ M memory in the SRBC system. These authors used, respectively, priming doses of  $5 \times 10^6$  and  $2 \times 10^6$  SRBC (in contrast to our dose of  $4 \times 10^8$  SRBC) and studied memory in an adoptive transfer system. Their results differ from one another. Cunningham found that  $5 \times 10^5$  transferred cells from primed donors gave a "greatly increased PFC response" per spleen in the irradiated recipients as compared to cells from unprimed donors. He also found that "ten to thirty-fold less cells (from primed donors) were required to give a significant response" in the irradiated recipients. This apparent memory was reported to persist with "no more than a hint of decline" for at least 5 months. The results of Shearer et al. did not indicate  $\gamma$ M memory in a similar system 4–5 months after priming.

At 7–10 days after immunization,  $\gamma$ G<sub>2a</sub> PFC have a considerably higher average density than the memory cells for this class. Using the relation between density and antigenic stimulation discussed above, we would suggest that the  $\gamma$ G<sub>2a</sub> memory cells arise later with respect to antigen administration than the  $\gamma$ G<sub>2a</sub> PFC. At the same time after immunization, other  $\gamma$ G memory cells (presumably  $\gamma$ G<sub>1</sub>) are not depleted from the denser gradient fractions. The physical separation of memory cells for two immunoglobulin classes shows that different cells carry memory for different classes. This conclusion is in agreement with that reached by Hamaoka et al. (36) and Shearer et al. (24). Our data is also in agreement with Hamaoka et al. (36) in showing that  $\gamma$ G<sub>1</sub> memory arises prior to  $\gamma$ G<sub>2</sub> memory. This order of memory cell appearance is the same as that reported by Wortis et al. for PFC of these classes (19).

The relationship between density and time after immunization is emphasized by the density changes in  $\gamma$ G<sub>2a</sub> memory seen over a period of 16 wk (Fig. 5). Soon after immunization, the  $\gamma$ G<sub>2a</sub> memory cells have a low density that increases progressively with time. By 6 weeks the difference in memory exhibited by the A and D fractions is no longer observed. Thus the memory cells have become similar in density to the general spleen and lymph node cell populations.

Cunningham (25) showed that primed spleen cell suspensions from which  $\gamma$ M PFC had been removed by micromanipulation, lost none of their ability to transfer a  $\gamma$ M PFC response. We have confirmed these results for  $\gamma$ M by using glass bead columns to remove virtually all  $\gamma$ M PFC from such suspensions without diminishing the ability of these suspensions to transfer a  $\gamma$ M response. Further, we have shown that antibody secreting cells are not needed for  $\gamma$ G memory. In fact, greater numbers of  $\gamma$ G PFC are obtained per  $10^6$  cells transferred after removal of  $\gamma$ G PFC by glass bead columns. This last finding may be due to avoidance of suppression by anti-SRBC antibodies that would have been released by transferred PFC. (37).

The methods used here for obtaining suspensions either greatly enriched or

depleted in memory cells will be useful in studies of cell interactions in the secondary response. This will be the subject of the subsequent paper of this series.

#### SUMMARY

Plaque forming cells (PFC) of different immunoglobulin classes producing antibodies against sheep erythrocytes were separated according to their buoyant densities by means of equilibrium centrifugation in a stepwise BSA gradient. In the period of 7–10 days after immunization  $\gamma$ M PFC are markedly enriched in fractions of low density and relatively depleted in fractions of high density. The distribution of total  $\gamma$ G PFC shows less enrichment in the lower density fractions and less depletion in the higher density fractions. The density profile for  $\gamma$ G<sub>2a</sub> PFC is even flatter, with a significant difference (depletion) relative to the unseparated spleen cells only in the highest density fraction.

The density gradient distributions of cells able to transfer an adoptive immune response of the various immunoglobulin classes are markedly different from the PFC distribution.

Cells obtained 7–10 days after immunization able to transfer an IgM response are present in the same proportions across the density gradient, whereas memory cells for  $\gamma$ G<sub>2a</sub> obtained at this time are markedly enriched in fractions of low density and virtually depleted from high density fractions. With increasing time after primary immunization, the  $\gamma$ G<sub>2a</sub> memory cells increase progressively in density and by 6 weeks the higher and lower density fractions have the same proportions of  $\gamma$ G<sub>2a</sub> memory cells. The total  $\gamma$ G (mainly  $\gamma$ G<sub>1</sub>) memory cells by 7–10 days show slight enrichment in low density fractions and no depletion in high density fractions. The conclusions were reached that (a) memory for  $\gamma$ G<sub>1</sub> develops earlier than memory for  $\gamma$ G<sub>2a</sub> and (b) that memory for anti-SRBC antibodies of different classes is carried in separate cells.

When gradient fractions enriched for PFC and memory cells for all classes were completely depleted of PFC using glass bead columns, the ability of this fraction to transfer memory for all classes was not diminished. This shows that memory cells are not identical with cells secreting antibodies.

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