CELLULAR DIFFERENTIATION OF THE IMMUNE SYSTEM OF MICE

I. Separate Splenic Antigen-Sensitive Units for Different Types of Anti-Sheep Antibody-Forming Cells*

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Upon primary injection of sheep red blood cells into mice, distinct populations of immunocytes secreting specific antibodies appear in the lymphoid organs and in the peripheral blood within days (1–6). Different types of immunocytes are characterized by the distinctive properties of the antibody they produce, such as the ability to lyse erythrocytes *directly* or *indirectly* upon facilitation (7–9), or to *agglutinate* erythrocytes rather than to *lyse* them (1, 4, 10). Also, the kinetics of production of each population of immunocytes so defined are different (2–4). The cells which release antibody arise from the proliferation and maturation of relatively immature or undifferentiated[1] precursors not producing antibody themselves but capable, in some way, of recognizing and reacting with antigen (3, 11–14). The antigen-sensitive cells found in the spleen and lymph nodes may be units of more than one cell, each of which could be specialized for a particular function, e.g., processing of antigen (15), reactivity with effective antigen (16), and/or production of cells which eventually synthesize immunoglobulins (16, 17). Irrespective of the number of components, such an antigen-sensitive unit may differentiate into antibody-forming cells of more than one kind, or into a single type of immunocyte.

A number of striking differences have been observed between the production of IgM and IgG antibodies and of lytic and agglutinating antibodies during the primary and secondary immune responses. These differences are (*a*) the dose of antigen required for induction and maintenance of IgM and IgG antibody production (18, 19); (*b*) the

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[1] Differentiation, acquisition of new properties by cells, i.e., qualitative change. Maturation, intensification of preexisting properties of cells, i.e., quantitative change.

kinetics of production of antibodies (18–20) and of the cells releasing them (2, 3); (*c*) the retention of immunological memory (18, 19); and (*d*) the sensitivity of the immune responses to prior X-irradiation (19–21). All these observations have raised the question of whether the different classes of antibodies are synthesized by cells derived from distinct populations of precursors, i.e., by separate unipotent antigen-sensitive units. An answer to this question could not be derived from the listed observations on the IgM and IgG responses, because most, if not all, of these observations could be explained also by assuming that pluripotent antigen-sensitive units undergo differentiation along two or more pathways. Different requirements for antigen, kinetics of cell proliferation, and recovery after irradiation could be associated with each pathway and with its induction. Hence, the question of the potentiality of antigen-sensitive units engaged in responses to a given antigen complex, although debated, has yet to be submitted to experimental test.

In the present work, some of the properties of splenic antigen-sensitive units were studied on a cellular level. Spleen cells of unprimed donor mice were transplanted with sheep erythrocytes into heavily irradiated syngeneic mice to quantitate the primary immune responses elicited by the grafted cells in terms of direct hemolytic plaque-forming cells (1), indirect hemolytic plaque-forming cells (8, 9), and agglutinating cluster-forming cells (4, 10). The validity of this approach rests on the fact that host animals have been rendered immunologically incompetent by the previous X-irradiation, and that cellular immune responses are a linear function of the number of spleen cells grafted (11). The sequential pattern in the production of the three types of immunocytes, and the dissociation of the responses in relation either to the number of cells transplanted or to the age of donor mice, were assessed 3–35 days after the grafting and stimulation of antigen-sensitive units. We have ascertained that these units have undergone extensive differentiation in the mouse spleen prior to the stimulation with sheep erythrocytes, such that most are already committed to produce a given population class of immunocyte upon further stimulation by antigen.

Materials and Methods

Mice.—(C3H/He × C57Bl/Ha)F_1 females, 11–14 wk old, were used in all experiments, except when the age of donor and recipient mice was an experimental variable.

Irradiation.—The prospective recipient mice were exposed to 700–900 R of X-radiation (250 kv peak, 30 ma, HVL (half-value layer) of 0.95 mm of Cu, source to target distance 50 cm, and exposure rate of 190 R/min.) in plastic centrifuge tubes attached to a Lucite turntable. Exposure measurements were made in air with a calibrated Victoreen R-meter.

Cell Suspensions.—Spleen cells were harvested by piercing the capsule with a sharp 18 gauge needle, then expressing the cells with blunt forceps by applying pressure perpendicular to the long axis of the spleen, while gently flushing the organ with Eagle's medium. Particulate matter was removed from the cell suspensions by filtering through 200 mesh/inch stainless steel cloth. Nucleated cell counts were made with a model B Coulter electronic particle counter fitted with a 100 μ aperture. The cell suspensions were adjusted to contain the desired number of spleen cells in 0.5 ml volume.

Transplantation and Immunization.—Spleen cell suspensions were mixed with an equal volume of sheep erythrocytes (SRBC) suspended in Eagle's medium immediately before transplantation. The SRBC were washed three times, counted with the Coulter apparatus, and adjusted to a concentration of 10^9/ml. Except when otherwise stated, each recipient mouse received 1 ml of the spleen cell–SRBC mixture, i.e., 5×10^8 antigenic sheep cells with variable numbers of potentially immunocompetent spleen cells. The cell mixture was injected into one of the lateral tail veins, within 4 hr after irradiation of the recipient mice. To prevent embolism, the mice received 50 USP units of heparin a few minutes before infusion of the cells.

Assay for Plaque-Forming Cells (PFC).—The direct hemolytic plaque test, which presumably detects immunocytes releasing 19S or IgM antibody, was carried out according to the method of Jerne et al. (1), except that plates were incubated at 37°C in a 5% CO_2 humidified atmosphere. The indirect hemolytic test for the detection of immunocytes, presumably releasing 7S or IgG antibodies, was carried out according to Wortis et al. (2), except that plates were incubated with guinea pig complement at 37°C for 4 hr. Goat-anti-mouse–IgG antiglobulin was purchased from Hyland Laboratories, Los Angeles, Calif., and used at a predetermined optimal dilution. Antiglobulin preparations partially inhibited the development of direct plaques. Corrections were necessary, therefore, to calculate the number of indirect plaques developed, since in each plate the total number of plaques detected included indirect as well as the fraction of direct plaques that developed in the presence of antiglobulin sera. Correction factors were calculated according to Wortis et al. (2).

Plaque assays were performed on spleen cell suspensions of individual mice. The assays were carried out in duplicate or triplicate plates with two or three serial cell dilutions. The average of all counts, expressed as plaque counts per unit number of spleen cells, was multiplied by the total number of cells harvested from the spleen (in multiples of 10^6) to estimate the number of PFC per spleen.

Assay for Cluster-Forming Cells (CFC).—The test was performed according to the description given by Zaalberg et al. (10), except for minor modifications. In brief, spleen cells were washed three times in Eagle's medium, adjusted to a concentration of 10^7 cells/ml, and incubated with SRBC (2% by volume) at 37°C for 75 min. After incubation, the spleen cell–SRBC mixtures were agitated to resuspend the cells and the newly formed clusters of erythrocytes surrounding single spleen cells. Presumably, such spleen cells released hemagglutinating antibody. Aliquots of the suspension were pipetted into Speirs-Levy slides to count, under microscopic examination, the clusters in a given volume of suspension. We regarded as clusters only large accumulations of tightly packed erythrocytes resembling a morula. We did not include in our counts spleen cells surrounded by less than 15 erythrocytes or aggregated spleen cells with or without SRBC. Each spleen cell suspension was examined in two to four counting slides. The average number of clusters per slide was multiplied by 12.5 to estimate the number of clusters per 10^6 spleen cells incubated. In turn, the latter number was multiplied by the total number of cells harvested from the spleen (in multiples of 10^6) to estimate the number of CFC per spleen.

Results of extensive work by Biozzi et al. (4, 22, 23), Zaalberg et al. (10, 24), and by us (unpublished observations) have shown conclusively that, under appropriate conditions, clusters of heterologous erythrocytes around splenic immunocytes detect cells actively engaged in the synthesis of agglutinating antibody, rather than cells coated by the specific antibody (passive sensitization) or by cytophilic antibody.

Statistical Methods.—The Poisson model was used to describe the theoretical probability that an inoculum of a given number of spleen cells would produce a positive recipient spleen for immunocytes measured by a particular assay. It is assumed that each competent cell to be transplanted has a given probability (P) of producing a positive spleen. As the number of cells in the inoculum becomes large, the probability that a recipient spleen will be positive approaches $1 - e^{-PD}$. When N_i spleens are assayed with an inoculum D_i, the probability that X_i of

them will be positive can be described by the binomial probability function with parameter $1 - e^{-PD_i}$ for each of m different inoculum sizes. The product of these m binomial functions is the likelihood function of the data. The method of maximum likelihood (25) was used to estimate the value of P. A search procedure was used to find for each limiting dilution assay the value of P which gave the highest value of the likelihood function. The 95% confidence intervals of P were calculated by varying the value of P until the natural logarithm of the likelihood function was smaller than the maximum by a critical value of 1.92. The procedure

TABLE I

Antibody-Forming Cells in Irradiated Control Animals

Treatment	Time after X-rays	Fraction of spleens with detectable immunocytes and mean number of immunocytes per positive spleen ± standard error		
		Direct PFC	Indirect PFC	CFC
	days			
SRBC	7–10	13/13 13.2 ± 4.5		5/11 3,170 ± 826
	11–14	3/11 35.5 ± 28.4	1/11 4.8	3/11 1,920 ± 240
Spleen cells ($5 - 50 \times 10^6$)	7–10	21/22 49.1 ± 10.5	0/11 —	9/15 10,500 ± 4,760
	11–14	4/4 52.1 ± 7.1	1/4 17.1	8/11 11,300 ± 1,670
Bone marrow cells ($5 - 50 \times 10^6$) + SRBC	7–10	4/4 41.0 ± 33.9	0/4 —	2/6 8,700 ± 5,730
	11–14	23/35 28.0 ± 5.5	11/15 19.2±3.9	4/12 13,500 ± 5,500

is based on a theorem describing the large sample distribution of the natural logarithm of the likelihood function (26).

RESULTS

Response in Control Mice.—Natural anti-sheep immunocytes are known to occur in the spleens of intact mice. Relatively small numbers of background PFC and CFC were also found in irradiated mice at varying times after injection of SRBC without spleen cells, or spleen cells without SRBC, or bone marrow cells (a tissue containing few, if any, antigen-sensitive units) with SRBC (Table I). None of the control mice had more than 100 direct PFC, 50 indirect PFC, and 2×10^4 CFC per spleen.

Response in Irradiated Mice Grafted with 2×10^7 Spleen Cells.—

A large group of heavily irradiated mice was given the mixture of spleen cells and SRBC by intravenous injection. Control groups received either spleen cells alone or bone marrow

cells instead of spleen cells, with SRBC. Assays for direct and indirect PFC and for CFC were carried out daily on individual spleens of 78 mice, from the 3rd to the 19th day after transplantation. The results obtained are shown in Figs. 1 and 2.

The spleens of *all* 78 mice contained direct PFC; the number of direct PFC increased between days 3 and 6 and then decreased slowly and irregu-

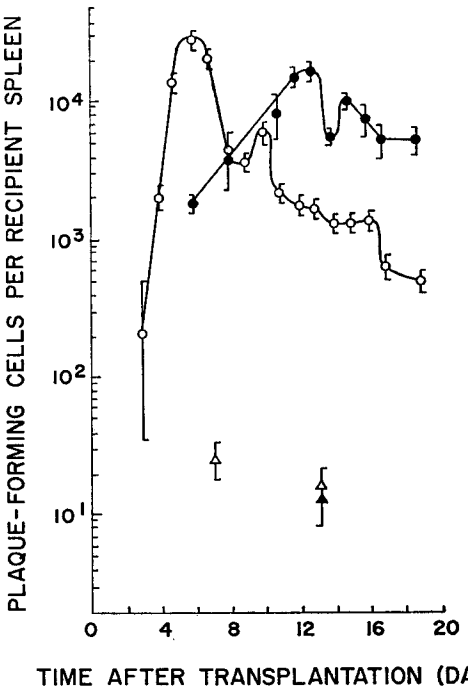

FIG. 1. Mean number of plaque-forming cells in spleens of irradiated mice at varying intervals after the injection of 2×10^7 spleen cells and 5×10^8 SRBC. Each point is the mean of 3–9 spleens assayed individually. Limits shown are two standard errors. ○, direct PFC in experimentals; ●, indirect PFC in experimentals; △, direct PFC in controls; ▲, indirect PFC in controls.

larly. The spleens of *all* 60 mice assayed between days 5 and 19 also contained indirect PFC. The number of indirect PFC increased between days 5 and 12, but did not decrease significantly thereafter.

In contrast to the PFC response which took place in all mice given potentially competent cells and antigen, the CFC response did not take place in all of the 40 mice assayed between days 7 and 19. Each of the groups of mice killed 7, 11, 13, 14, 15, and 17 days after transplantation included a few animals whose spleens did not contain CFC above control levels. A total of 12 of 40 tested mice did not respond to primary SRBC stimulation with CFC, although

the mice responded with PFC. Mean values of CFC per spleen in positive mice only are shown in Fig. 2. These observations suggested that 2×10^7 grafted spleen cells contained enough antigen-sensitive units (hereafter abbreviated ASU) lodging in the recipient spleens to provide them with direct and

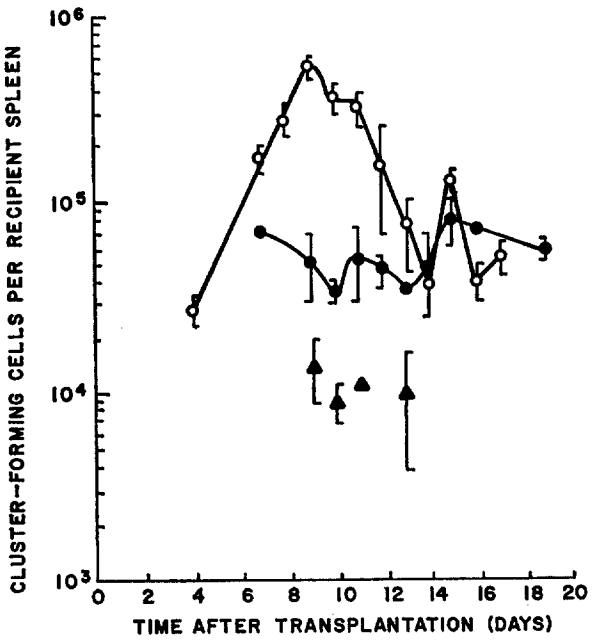

FIG. 2. Mean number of cluster-forming cells in spleens of irradiated mice at varying intervals after the injection of spleen cells and 5×10^8 SRBC. Each point is the mean of 2–5 spleens assayed individually. Limits shown are two standard errors. ○, 10^8 spleen cells grafted (30/30 positives); ●, 2×10^7 spleen cells grafted (28/40 positives); ▲, spleen cells without SRBC (controls).

indirect hemolysin-forming cells, but not enough units to also provide each recipient spleen with agglutinin-forming cells.

CFC Response in Irradiated Mice Grafted with 10^8 Spleen Cells.—

A group of 30 irradiated mice was grafted with 10^8 spleen cells and SRBC and assayed for CFC from days 4 to 17.

The spleens of all mice contained CFC (Fig. 2); their numbers increased from day 4 to day 8 and then decreased slowly. The number of CFC per spleen remained above that of CFC in control animals throughout. It appears, therefore, that precursors of CFC are transplantable, but that greater numbers of spleen cells are required to ensure production of CFC in every recipient spleen than to

ensure production of PFC. This observation is consistent with the possibility that separate antigen-sensitive precursors exist for PFC and CFC and that their frequency in donor spleen is different. Alternatively, differentiation of ASU into CFC may be more complex than differentiation into PFC and require, therefore, the more extensive host spleen repopulation provided by the larger grafts.

Response in Irradiated Mice Grafted with 5 × 10^6 Spleen Cells.—If there are separate and independent ASU, each of which is endowed with the potential to generate a single type of anti-SRBC immunocyte in recipient spleens, it should be possible to reduce the size of spleen cell grafts so that a given inoculum contains only one or two types of ASU. If this hypothesis is correct, limiting dilution assays should provide an estimate of the number of ASU in spleen cell suspensions. The procedure consists in finding the number of spleen cells mixed with SRBC which, on transplantation into a large group of animals, yields positive responses in some members and no response in others of the same group. Statistical methods based on the Poisson distribution are expected to be applicable to experimental data of this type. Kennedy et al. (11) and Brown et al. (14) have already used limiting dilution assays to enumerate antigen-sensitive precursors of direct PFC and of hemagglutinating antibody in the mouse. It was necessary, therefore, for us to establish whether indirect PFC and CFC produced by few precursor units, i.e. by small grafts, are detectable in a spleen cell transfer system.

5 million spleen cells mixed with 5 × 10^8 SRBC were injected into a series of mice. 9–12 days later, at the time of expected peak responses for indirect PFC and CFC (Figs. 1 and 2), two-thirds of the mice were killed and their spleens were assayed for direct and indirect PFC. Some of the spleens were also assayed for CFC. The other third of the grafted mice were killed 30–35 days after transplantation and their spleens were assayed for all three types of immunocytes.

The spleens of almost all mice killed at the earlier interval (56 of 63) were positive for direct PFC; this indicates that they had been effectively immunized and that the grafts were immunologically competent. Furthermore, most of the spleens of mice killed at the later interval (22 of 28) contained more direct PFC than control animals, but the number of PFC was relatively low, as expected (Fig. 1).

The results of assays for indirect PFC and CFC are shown in Figs. 3 and 4, respectively. The observed frequencies of the different values of immunocytes per recipient spleen are plotted. One group of spleens can be identified with indirect PFC and CFC values not different from those of control mice (up to 50 indirect PFC and 2 × 10^4 CFC), and a second group with values significantly greater than those of controls. We regard the former spleens as negative and the latter as positive. Negative spleens were not due to delay in the time of peak responses because they were found to be negative in about the same

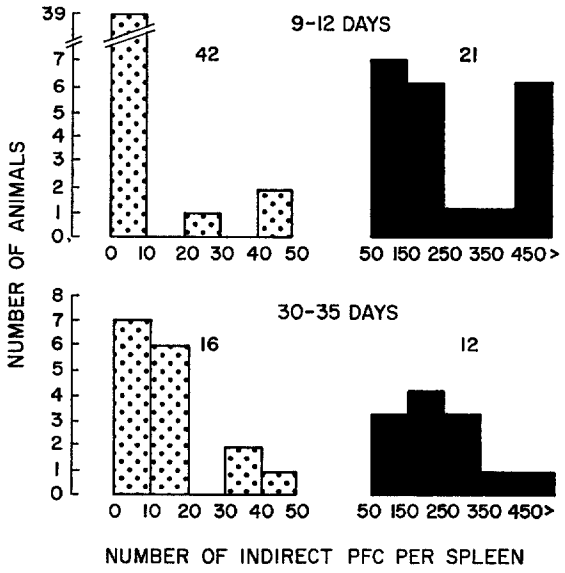

FIG. 3. Number of animals from which spleens assayed 9–12 or 30–35 days after antigenic stimulation were negative (0–50 or radiation control values) or positive (>50) for indirect plaque-forming cells. Each animal was exposed to 900 R of X-rays and grafted with 5×10^6 spleen cells mixed with 5×10^8 SRBC.

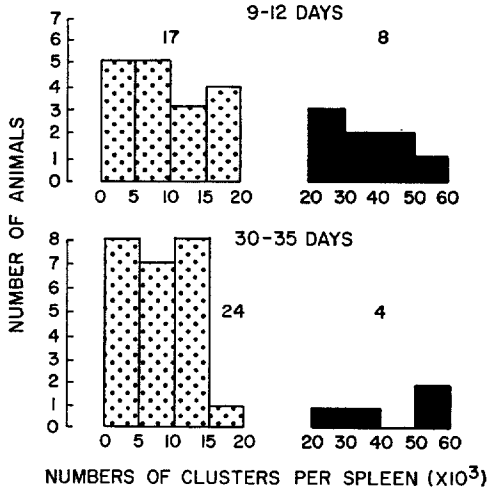

FIG. 4. Number of animals from which spleens assayed 9–12 or 30–35 days after antigenic stimulation were negative (0–20 $\times$ 10^3 or radiation control values) or positive (>20 $\times$ 10^3) for cluster-forming cells. Each animal was exposed to 900 R of X-rays and grafted with 5×10^6 spleen cells mixed with 5×10^8 SRBC. Each spleen was also assayed for indirect plaque-forming cells (see preceding figure).

proportion 30–35 days after transplantation, and also at intermediate intervals (data not shown in Figs. 3 and 4). It is conceivable that the negative spleens belonged to mice in which not even one grafted ASU lodged in this organ.

5 million spleen cells were mixed with 5×10^7 SRBC instead of 5×10^8 and the mixture was injected into 8 irradiated recipients. This was done to exclude the possibility that lack of response was due to excess antigen. The recipient spleens were assayed 10 days after grafting; all were positive for direct PFC, but only 4 of 8 were positive for indirect PFC, and none were positive for CFC.

It appears that ASU that can be assayed by transplantation exist not only for direct PFC (11), but also for indirect PFC and for CFC. The progeny of one or two ASU can be readily detected by the two assays. This is so in spite of the fact that the efficiency of the plaque assay is greater than that of the cluster assay. The total number of spleen cells examined for the presence of immunocytes is 8×10^4 per counting slide (cluster assay), as opposed to $5 - 15 \times 10^6$ per plate (plaque assay). The experiments described above did not measure the frequency of the ASU, but it appears that precursors of the three types of immunocytes studied are not equally frequent in spleens of unprimed mice, and that the order decreases from precursors of direct PFC, to those of indirect PFC and CFC.

Limiting Dilution Assays.—

In a large number of experiments, groups of irradiated mice received increasing numbers of spleen cells mixed with SRBC. After 7–9 days a total of 270 individual spleens of survivors were assayed for their content of direct PFC. Another 201 mice were assayed on days 8–11 for splenic CFC, and 333 mice on days 11–14 for splenic indirect PFC. Each type of cellular immune response was measured, therefore, at the time of its anticipated peak value (Figs. 1 and 2). Spleens were regarded as positive if the number of immunocytes exceeded 100 direct PFC, 50 indirect PFC, and 2×10^4 CFC. Otherwise, the spleens were regarded as negative. The results are presented in Table II.

As the number of grafted cells increases from 0.12 to 5×10^6, the proportion of mice with spleens positive for direct PFC also increases. Virtually all spleens were positive when 3×10^6 spleen cells from unprimed donors were grafted. However, grafts of 3×10^6 or fewer cells did not yield as many positive spleens for indirect PFC and CFC. This indicates that under these circumstances ASU were competent to produce direct PFC only. The inoculum sizes which yielded increasing proportions of mice with spleens positive for indirect PFC ranged from 0.5×10^6 to 4×10^7. These inoculum sizes still left a proportion of mice with spleens negative for CFC. It follows that larger grafts contain ASU competent to produce indirect PFC (and perhaps also direct PFC), but not always CFC. To obtain this type of cellular immune response consistently, it was necessary to further increase the inoculum size, up to 10^8 spleen cells per recipient mouse. In conclusion, in each group of mice, the spleens of some

recipients were positive in that they contained significant numbers of antibody-forming cells, while others were negative. But the relation between the percentage of positive spleens and the number of potentially competent cells grafted varied greatly for each type of antibody-forming cell studied.

TABLE II

Percentage of Recipient Spleens Containing Significant Numbers of Immunocytes as a Function of Spleen Cell Inoculum Size

Type of immunocyte	Cells transplanted ($\times 10^6$)	Fraction of spleens containing significant numbers of immunocytes*	Percentage of positive spleens
Direct PFC	0.12	3/29	10.3
	0.25	2/13	15.4
	0.50	16/39	41.0
	1.0	31/48	64.5
	2.0	54/60	90.0
	3.0	17/18	94.5
	5.0	62/63	98.4
Indirect PFC	0.50	3/14	21.2
	1.0	5/32	15.6
	2.0	12/41	29.3
	3.0	9/18	50.3
	5.0	26/69	37.7
	10.0	40/54	74.0
	15.0	30/32	93.7
	20.0	55/57	96.5
	40.0	16/16	100.0
CFC	0.50	0/18	0
	1.0	3/28	10.7
	2.0	4/18	22.2
	5.0	9/32	24.3
	10.0	3/11	27.3
	20.0	12/21	57.1
	25.0	17/21	81.0
	30.0	11/13	84.6
	100.0	33/34	97.0

* More than 100 direct PFC, 50 indirect PFC, 2×10^4 CFC, per spleen.

The statistical methods described in Materials and Methods were applied to the limiting dilution data of Table II for each type of immunocyte to estimate P, the probability value per 10^6 grafted cells that an inoculum provides the recipient spleen with ASU capable of generating detectable numbers of antibody-producing cells. The estimates of P and their 95% confidence intervals are presented in Table III. The curves relating inoculum size to the expected frequency of positive spleens are shown in Fig. 5, together with the observed frequencies. Results of this analysis provide an estimate of the number of grafted ASU which

reached the recipient spleens. According to other investigators (11, 12) only a small fraction of ASU injected into the tail vein of irradiated mice do reach the spleen, i.e., ~15% if measurements are made 2 hr after transplantation (11) or ~4% if the measurements are made 24 hr after transplantation (12). We have not corrected the values of Table III for dilution of ASU in the recipients because of time-dependence of the value of correction factors. Furthermore, it is not known whether the correction factors would be equal for every type of ASU. All the data presented thus far suggest that ASU interacting with SRBC are heterogeneous. It is evident from the analysis presented in Table III that our method of assay detects about one ASU for direct PFC in 0.81 — 1.2 × 10^6 spleen cells, whereas one ASU for indirect PFC or for CFC was detected in 5.8 — 8.2 × 10^6 and 14.8 — 25 × 10^6 spleen cells, respectively. The fact that none of the 95% confidence intervals overlap indicates that these differences are statistically significant.

TABLE III

Results of Statistical Analysis of Limiting Dilution Assays: Calculated Frequencies of Antigen Sensitive Units in Spleen Cell Suspensions of Young Adult Unprimed (C3H × C57Bl)F_1 Female Mice*

	Type of immunocyte assayed		
	Direct PFC	Indirect PFC	CFC
Probability of positive spleen per 10^6 cells injected	1.02 (0.84–1.23)‡	0.14 (0.12–0.17)	0.052 (0.040–0.068)
No. of spleen cells (× 10^6) containing one detectable antigen-sensitive precursor	0.98 (0.81–1.19)	6.90 (5.85–8.17)	19.3 (14.8–25.0)

* Only antigen-sensitive units which reach the spleens of recipient mice (4–15% of all antigen-sensitive units in the inoculum).

‡ 95% confidence intervals in parentheses.

The sensitivities of the two plaque assays and of the cluster assay depend on the number of antibody molecules necessary to detect a single immunocyte. It is difficult, therefore, to assess the comparative sensitivities of the assays, so as to exclude that the results obtained in limiting dilution experiments reflected decreasing sensitivities of methods rather than decreasing frequencies of ASU. According to Humphrey and Dourmashkin (27), red blood cells require about a thousand times more IgG than IgM molecules for lysis *without facilitation*. If this reduces significantly the sensitivity of the indirect plaque assay (*with facilitation*) below that of the direct plaque assay, it might well be that the frequency of ASU for indirect PFC is higher than that indicated in Table III. The frequency determinations of ASU for CFC may be on firmer ground. According to Zaalberg et al. (24), relatively few molecules of antibody are necessary for cluster formation, considerably fewer than those necessary for plaque formation (1). Hence, the displacement of the CFC limiting dilution curve to the right (Fig. 5) occurred even though the sensitivity of the assay was,

presumably, the greatest. For these considerations, we regard the ASU frequency values found in limiting dilution assays (positive vs. negative spleens) as relative numbers reflecting true differences, although we are aware that the absolute numbers of ASU and antibody-forming cells may depend in part on assay sensitivity. Future progress in this area will elucidate this point.

If ASU are restricted in their potential for differentiation to a single type of functional progeny cell, and, furthermore, reach the spleens of recipients inde-

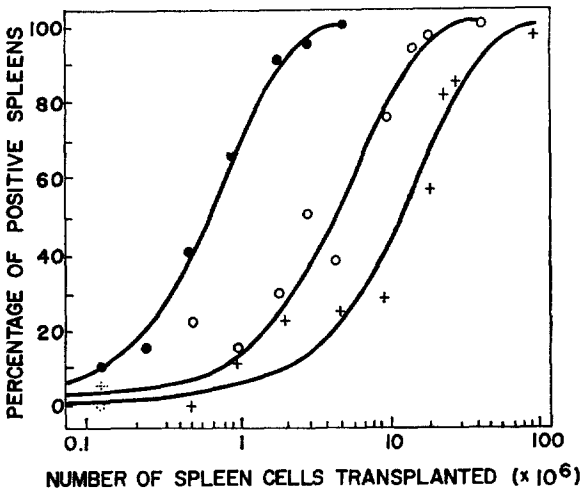

FIG. 5. Percentage of recipient spleens positive for antibody-forming cells following X-irradiation and injection of graded numbers of spleen cells mixed with 5×10^8 SRBC. The number of spleens assayed for each inoculum size is given in Table II. Curves indicate expected percentages and symbols observed percentages. ●, direct plaque-forming cells assayed on days 7–9; ○, indirect plaque-forming cells assayed on days 11–14; +, cluster-forming cells assayed on days 8–11; broken circle and plus sign, data obtained after statistical analysis was completed.

pendently, then we expect to find occasional spleens that are positive for the progeny of the more infrequent ASU, but negative for the progeny of the more frequent precursor. To test this possibility, we surveyed our protocols for irradiated mice injected with spleen cell-SRBC mixtures, assayed for all three types of splenic immunocytes. We found among approximately 150 mice, 6 whose spleens contained direct PFC and CFC, but not indirect PFC (Table IV). This indicates unequivocally that the precursors of CFC and of indirect PFC are different. However, we did not find at the time of peak response recipient spleens positive for indirect PFC but negative for direct PFC. This is probably due to the greater difference in frequency between these two ASU. Consequently, we cannot exclude that ASU capable of generating indirect PFC also

generate direct PFC. However, the most frequent splenic ASU we studied were unipotent, for they generated in a number of occasions direct but not indirect PFC (Fig. 5).

TABLE IV

Presence of Cluster-Forming Cells in Spleens Lacking Indirect Plaque-Forming Cells

Spleen cells transplanted ($\times 10^6$)	Time after X-rays	Immunocytes per spleen		
		Direct PFC	Indirect PFC	CFC
	days			
1	10	270	0*	92,600
2	9	558	0	36,200
5	8	474	0	54,200
5	34	250	47	65,300
10	8	390	0	36,500
50‡	13	571	5	94,000

* Less than one PFC in $10 - 30 \times 10^6$ recipient spleen cells plated.

‡ Donor's age, 8 days.

TABLE V

Antibody-Forming Cells in Irradiated Mice Injected with 5×10^7 Spleen Cells from 8 Day Old Donors and 5×10^8 SRBC

Age of host	Time after X-rays	Fraction of spleens with detectable immunocytes and mean number of immunocytes per positive spleen ± standard error		
		Direct PFC	Indirect PFC	CFC
wk	*days*			
2	7–10	6/6 1620 ± 490	4/6 531 ± 377	
2	13	8/8 526 ± 83	7/8 380 ± 40	3/8 46,300 ± 22,100
13	7–8	6/6 2370 ± 1150	2/6 162 ± 81	
13	13	9/9 1360 ± 351	9/9 1940 ± 662	6/9 57,000 ± 14,100

Response in Irradiated Mice Grafted with Spleen Cells from Infant Donors.—It has been reported that humoral antibodies such as anti-SRBC hemagglutinins (28, 29) and allohemagglutinins (30) synthesized by 3–20 day old mice were solely mercaptoethanol-sensitive or of the macroglobulin type. As mice became older, mercaptoethanol-resistant or 7S-type gamma globulins were also pro-

duced. It was implied that infant mice were immature and did not possess antigen-sensitive precursors of immunocytes synthesizing mercaptoethanol-resistant antibodies (28, 29). If this were so, immature infant mice should not possess precursors of indirect PFC and of CFC releasing IgG antibodies. Such infant mice prior to maturation would provide a source of unipotent ASU,

TABLE VI

Antibody-Forming Cells in Adult Irradiated Mice Injected with 10^8 Spleen Cells from 4 to 17 Day Old Donors and 5×10^8 SRBC

Age of Donor	Time after X-rays	Fraction of spleens with detectable immunocytes and mean number of immunocytes per positive spleen ± standard error		
		Direct PFC	Indirect PFC	CFC
days	*days*			
4	7	2/2 2020	2/2 343	
4	13	5/5 2280 ± 380	4/5 659 ± 200	1/5 21,300
8–9	7	2/2 4390	1/2 1020	
8–9	13	4/4 1240 ± 330	4/4 3030 ± 638	3/4 70,300
10–12	8	2/2 1480 ± 20	2/2 308 ± 46	
10–12	13	3/3 1270 ± 825	3/3 2720 ± 964	3/3 222,000 ± 33,600
15–17	7–9	9/9 3090 ± 814	9/9 2220 ± 335	2/9 64,800 ± 11,000
15–17	10–11	9/9 2160 ± 250	9/9 9300 ± 3000	9/9 72,900 ± 13,200

naturally separated one from the other. Furthermore, the adult recipient spleen after cell transfer could be viewed as being similar to that of an intact infant mouse, characterized by rapid expansion and differentiation of a small population of potentially immunocompetent cells.

We harvested the spleens of several 8 day old mice, dispersed the cells, and mixed them with SRBC. Each donor spleen contained approximately 2×10^7 nucleated cells; 5×10^7 spleen cells per recipient were injected into 2 wk old and into adult irradiated mice. The former

were exposed to 700 R instead of 900 R of X-rays, and received their grafts by tail vein injection in 0.25 ml volumes. Results of spleen assays for immunocytes are presented in Table V.

Pooled spleen cell suspensions of infant donors contained precursors of indirect PFC and of CFC which were detected both in infant and in adult hosts. Spleen cells from another group of infant mice, 4 to 17 days old, were transferred into adult hosts only (Table VI). Pooled cell suspensions from 4 day old donors (10^8 cells per recipient) already contained ASU for all three types of immunocytes. The results with spleen cells taken from infant donors were qualitatively comparable to those obtained with spleen cells taken from adult mice, but the frequency and total number of ASU were considerably lower in infants. The reported failure to detect 7S-type agglutinins in serum (28) may well be explained by the small number of lymphoid cells in infant mice and by the lower frequency of the relevant ASU. The use of infant mice did not provide, therefore, the means for separating different types of ASU nor for studying the effect of developmental changes on their differentiation.

DISCUSSION

It has been demonstrated by Trentin et al. (31) that cells contained in 4–13 marrow-derived hemopoietic colonies can establish, in irradiated mice, a large population of immunocompetent cells reactive to a variety of unrelated antigens. It follows that marrow is the source of undifferentiated progenitor cells of the immune system. Such progenitor cells are yet incapable of reacting with antigen (5, 16, 32), but generate, *via* thymus-dependent differentiation, antigen-sensitive units (32, 46) and cells synthesizing antibody (16). This pathway of differentiation produces an extremely specialized and heterogeneous population of end cells, i.e., immunocytes releasing, as a rule, one of a number of molecular species of antibody (33).

The specialization conferred upon immunocytes results (*a*) in antibody production against a single antigenic determinant, i.e., in specificity differentiation (6, 12, 13, 22, 23); (*b*) in production of a single class of antibody molecule among several directed against the same antigen, i.e., in class differentiation (6, 34, 35); (*c*) in production of one allotypic variant of a given molecular species of antibody, i.e., in allotypic restriction (36, 37). We have found that cells releasing anti-sheep hemolysins and hemagglutinins are specialized and that they belong to separate populations. Some spleens containing $>36{,}000$ cluster-forming cells, were lacking indirect plaque-forming cells (Table IV), and spleens that contained direct plaque-forming cells were lacking, under given circumstances, cluster-forming cells (Figs. 2 and 4). Furthermore, we have verified (unpublished observations) that cluster-forming cells incubated first in liquid medium with SRBC (where they actually formed clusters) and then suspended in agar medium did not also produce direct nor indirect hemolytic plaques. This

confirms observations reported by Zaalberg et al. (10), but is at variance with a subsequent communication by the same authors (24). In the latter case, plaque formation was studied using monolayers in liquid medium. A number of plaque-forming cells ($\sim 12\%$) were seen surrounded by large clusters of SRBC ghosts. This was interpreted to mean that such plaque-forming cells were also cluster-forming cells. We suggest that these accumulations of SRBC ghosts may be artifacts, i.e., ghosts of SRBC that once filled the area of the plaque, but which have subsequently become adherent to the immunocyte at the center.

Sheep erythrocytes are a mosaic of antigenic determinants so that it is possible for different immunogens to elicit the agglutinin and lysin responses (9). Accordingly, cluster-forming cells and plaque-forming cells would appear as independent cell populations undergoing specificity differentiation. However, we cannot rule out class differentiation since subclasses of mouse IgG antibodies have been shown to mediate different biological activities (38, 39). For example, IgG1 antibodies were capable of hemagglutination but not of complement-dependent hemolysis, whereas IgG2 antibodies were capable of both activities. Experiments with simpler antigens, possibly with a single determinant, could resolve this question.

We have found unequivocal evidence for class differentiation among cells releasing IgM and IgG hemolysins. Some spleens that contained direct plaque-forming cells were lacking indirect plaque-forming cells at the time of peak response and up to 20 days later (Fig. 3 and Table IV). This is in agreement with the elegant observations of Celada and Wigzell (6) and of Brahmi and Merchant (35) on the exclusive secretion of either IgM or IgG hemolysins by mouse and rabbit immunocytes. If clonal populations of IgM-secreting cells were to shift to IgG secretion, as proposed by Papermaster (40) and by Sterzl (41), one would expect that each spleen containing direct plaque-forming cells would sooner or later also contain the indirect variety. We have failed to verify this expectation within 35 days after antigenic stimulation.

The major purpose of our experiments was not so much to verify the heterogeneity of antibody-forming cells, already established in several laboratories (6, 33–35), but rather to investigate whether this heterogeneity arose before or after interaction of antigen with antigen-sensitive units. Our findings clearly indicated that the precursors of antibody-forming cells were already heterogeneous and that they displayed in the spleens of adult and infant mice the same type of differentiation, i.e. class and/or specificity differentiation, detectable among their progeny cells. In other words, antigen-sensitive units were tooled up to generate either direct plaque-forming cells only, or indirect plaque-forming cells (with or without direct PFC), or cluster-forming cells only, prior to the administration of SRBC. Due to the occurrence of natural anti-SRBC antibodies in mice, we cannot exclude that differentiation of antigen-sensitive units was directed by cross-reacting antigens. Regardless of the nature of the control

mechanisms, we have established the existence of distinct and unipotent splenic precursors of antibody-forming cells. If the antigen-sensitive units were pluripotent at the time of SRBC administration, but were to become unipotent or committed immediately after contact with the relevant antigenic determinant, then measurement of their frequencies should have yielded equal values, regardless of the type of progeny immunocyte assayed. We found instead significant differences in the frequency of antigen-sensitive units measured by three types of immunocytes. The frequency values we found are in close agreement with those reported by Kennedy et al. (11) and by Brown et al. (14) for precursor units of direct plaque-forming cells and of serum hemagglutinin. We have already discussed in the Results section the influence of efficiency and sensitivity of the assay methods, amount of antigen, interval between antigen administration and assay, and size of the pool of potentially competent cells, on this interpretation.

The pluripotent nature of the primitive progenitor (of marrow origin) of immunocompetent cells (31) is lost when the progenitor has generated splenic antigen-sensitive units. Perhaps cells with intermediate properties of differentiation and maturation can be found in marrow-thymus mixtures (16, 17) or in fetal tissues. Differentiation may have occurred either in all cell components of an antigen-sensitive unit (15–17) or only in a single cell type such as macrophages processing antigen, thymocytes, or cells synthesizing immunoglobulins. Results of preliminary experiments suggest that antigen-sensitive units resulting from bone marrow-thymocyte mixtures are also unipotent with respect to anti-SRBC immunocyte production. Nossal et al. (42) have found that splenic antigen-sensitive units of mice responding to primary administration of SRBC and to primary and secondary administration of *Salmonella* flagellin have undergone specificity and class-differentiation so as to be physically separable. Presumably, differentiation of distinct potentially competent cells occurs during ontogeny, since rubella virus infections contracted in utero can impair specifically the functions of human IgG-producing cells and/or cell-bound immunity (43, 44). Long-lasting depression of IgG antibody formation follows X-irradiation, while IgM antibody formation resumes (19–21); this suggests, furthermore, that properties of class-differentiation are acquired early by potentially competent cells along their production pathway.

The broad spectrum of immunologic reactivity of mammals in general, and the heterogeneity of the antibody response, even when elicited by inbred mice against haptens,[2] may find its basis in cellular heterogeneity. If several cells endowed with distinct functions cooperate in establishing an antigen-sensitive unit, and if each cell type undergoes differentiation and maturation so as to become extremely specialized, the interplay and/or random association of such

[2] Matioli, C. A., Y. Yagi, and D. Pressman. 1968. Heterogeneity of antibodies produced by inbred mice. Manuscript submitted for publication.

cells, upon antigenic stimulation, could be a source of sufficient variability to account for the great heterogeneity of responses. If differentiation of potentially competent cells is antigen-independent, influences of the microenvironment, such as those postulated by Wolf and Trentin for myelopoietic cells (45), could be the stimulus for differentiation. These concepts are amenable to experimental test by the use of recently introduced techniques of cellular immunology.

SUMMARY

Spleen cell suspensions of unprimed donor mice containing precursors of immunocytes have been transplanted into X-irradiated recipient mice. In the presence of antigen (sheep erythrocytes) these precursors, called antigen-sensitive units, gave rise to progeny cells secreting specific antibody. We studied quantitatively the production of cells releasing IgM hemolysins (direct plaque-forming cells), IgG hemolysins (indirect plaque-forming cells), and hemagglutinins (cluster-forming cells). We found that each of these immunocyte populations was distinct, i.e., that cells releasing agglutinins did not, as a rule, release hemolysins, and vice versa. We also found that cell populations secreting IgM hemolysins did not shift, under certain experimental conditions, to the production of IgG hemolysins during the primary immune response.

By transplanting graded numbers of spleen cells, we succeeded in limiting to one or a few the number of antigen-sensitive units that reached the recipient spleen. We estimated thereby the frequency of antigen-sensitive units in donor cell suspensions and tested their potential for production of immunocytes of more than one type. Our results indicated that antigen-sensitive units were unipotent for they displayed in the spleens of unprimed donors the same restrictions of function and heterogeneity (antibody-specificity differentiation, antibody-class differentiation) found among antibody-forming cells. Furthermore, antigen-sensitive precursors for direct plaque-forming cells, indirect plaque-forming cells, and cluster-forming cells were detected in the spleens of unprimed mice in different frequencies, i.e., 1 in $\sim 10^6$, 1 in $\sim 7 \times 10^6$, and 1 in $\sim 19 \times 10^6$ spleen cells, respectively.

We concluded that relatively advanced differentiation of potentially competent cells occurs before sheep erythrocyte administration. The relevance of this finding for the broad spectrum of immunologic reactivities and for the heterogeneity of antibody responses to given antigens was discussed.

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