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Studies on the Cellular Basis of IgM Immunological Memory*

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Summary. (1) A direct experimental method has been used to test whether IgM immunological memory is carried by antibody-forming cells or by cells not releasing detectable antibody.

(2) An adoptive transfer system was used to demonstrate memory. Donor mice were injected intravenously (i.v.) with sheep erythrocytes, and at intervals thereafter, dispersed cells from their spleens were transferred to lethally irradiated recipient mice, together with more antigen. The greatly increased plaque-forming cell responses in the spleens of irradiated mice receiving primed as compared to unprimed donor cells was taken as a measure of the level of IgM memory in the donor mice.

(3) Suspensions of donor spleen cells were divided mechanically (by micromanipulation) into: (a) a plaque-forming cell fraction, and (b) a fraction totally free of plaque-forming cells. Each fraction was injected, with antigen, into an irradiated recipient mouse, and the response of these mice was compared with that of a third animal which received unfractionated cells. It was consistently found that irradiated mice receiving plaque-free populations of cells responded as well as the controls, while no response was observed in mice receiving donor plaque-forming cells alone. A similar approach showed that the antigen responsiveness of spleen cells from unprimed mice is independent of their content of 'background' plaque-forming cells. It is concluded that IgM immunological memory is carried by cells which are not producing plaques, and reasons are discussed for supposing that memory cells and antibody-forming cells belong to separate cell lineages.

INTRODUCTION

When an animal is exposed to a given antigen, it may produce an increased number of cells capable of responding specifically to restimulation with the same antigen (Albright and Makinodan, 1965). Various theories on the origin of these 'memory' cells have been advanced (Nossal, 1965; Albright and Makinodan, 1965; Sercarz and Byers, 1967) but it is not yet clear how their genesis is linked to the development of the antibody-forming-cell line.

Two observations suggest that the property of immunological memory might be carried

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by the antibody-forming cells themselves. First, it seems possible that memory cells have specific receptors for antigen on their surface (Monod, 1959), receptors which could well be antibody molecules. Antibody-producing cells would fall into this category of receptor-bearing cells. Second, several recent papers have described a very rapid development of memory after priming, with kinetics similar to the appearance of antibody or antibody-forming cells (Wigzell, 1966; Vischer, Stastny and Ziff, 1967; Sercarz and Byers, 1967). For example, Sercarz and Byers (1967) claimed that significant IgM memory to sheep red cells developed in mice as early as 1 day after priming, and found that this memory reached a peak at about a week after antigenic stimulation. Such a close correspondence between the generation of memory and of antibody-forming cells might be expected if the antibody-forming cells themselves were capable of further proliferation when restimulated with antigen.

This paper describes experiments which test directly whether IgM memory to sheep erythrocytes is carried by plaque-forming cells or by non-plaque-forming cells at different stages throughout the primary response in mice. Also tested is the proposition (Aisenberg and Wilkes, 1967), that cells which respond to sheep red cell antigen in unprimed mice are the background plaque-forming cells.

MATERIALS AND METHODS

Mice

In most experiments CBA-strain females were used; they were 8–14 weeks old at first injection. Within any one experiment, the variation in age was not more than 2 weeks. Males, and mice of the CBA/T6T6 strain (syngeneic with CBA), were used in a few experiments.

Irradiation

A deep X-ray machine, operating under conditions of 250 kV, 15 mA, 0·25 mm Cu, 1 mm Al filtration, and a focal skin distance of 30 cm with full backscatter conditions was used to irradiate mice, which generally received 850 rad.

Preparing spleen cell suspensions

Cells were always manipulated in Eisen's medium (Helmreich, Kern and Eisen, 1961), with 10 per cent added foetal calf serum. Spleens were gently rubbed on a piece of fine mesh nickel wire gauze under medium. Large clumps were removed by centrifugation at 100–200 ***g*** for a few seconds; the dispersed cells were then collected by spinning at 400 ***g*** for 5 minutes, then resuspended to the appropriate concentration, in fresh medium.

Assays for plaque-forming cells

A sensitive modification of the haemolytic plaque technique was used (Cunningham and Szenberg, 1968) to detect 19S (direct, IgM) plaques. No supporting medium was used, spleen cells and erythrocytes being incubated together in a monolayer at the bottom of a very shallow chamber. Indirect (7S IgG) plaques were scored by adding rabbit anti-mouse γ-globulin (final dilution 1 : 150) to the medium in some chambers (Sterzl and Riha, 1965; Dresser and Wortis, 1965).

Micromanipulation

To identify plaque-forming cells for micromanipulation, a mixture of spleen cells, target erythrocytes and complement was incubated at 37° for 25 minutes as a monolayer in

a series of droplets of medium under paraffin oil. While single plaque-forming cells could be readily isolated with a micropipette from such drops if a sufficiently dilute suspension of lymphoid cells was used (Cunningham, Smith and Mercer, 1966; Cunningham, 1968), many more plaques could be handled if the spleen cells were plated out at high concentration (about 2×10^7/ml). Under these conditions a droplet of volume 2 μl contained approximately 4×10^4 spleen cells, and a large plaque had up to 200 nucleated cells inside its boundaries. By pipetting up all these cells, the collection of the plaque-forming cell was assured. This procedure was used to assemble large numbers of plaque-forming cells, and it was established by direct counting that an average of not more than 200 nucleated cells was drawn into the pipette with each plaque-forming cell. By contrast, to obtain a population of cells deprived of all plaque-formers, spleen cells were plated out at such a dilution that one droplet in two had no plaques in it. All the cells from these plaque-free drops were then collected with a relatively coarse micropipette.

Assay for memory

An adoptive transfer system was used to demonstrate memory. Donor mice were injected i.v. with 5×10^6 sheep erythrocytes, then at various intervals dispersed-cell suspensions were prepared from the spleens of killed donors, and 5×10^5 of these spleen cells were injected into each of several syngeneic recipient mice which had been lethally irradiated 24 hours earlier, together with 2×10^8 sheep erythrocytes. The recipients were killed 7 days later, and their spleens assayed for plaque-forming cells. The greatly increased plaque-forming cell response in the spleens of irradiated mice receiving spleen cells from primed as compared to unprimed donors was taken as a measure of the level of memory in the donor mice.

Balance-sheet experiments

At all stages of the primary response of donor mice (Fig. 1), the question was asked: is the property of memory carried by plaque-forming cells or by non-plaque formers? For example, at 8 days after priming, the spleen of a normal mouse contains about 100 plaque-formers/10^6 cells. From Fig. 2 it can be seen that transferring 5×10^5 such primed cells to an irradiated mouse would give about 3000 plaques in the spleen of the recipient

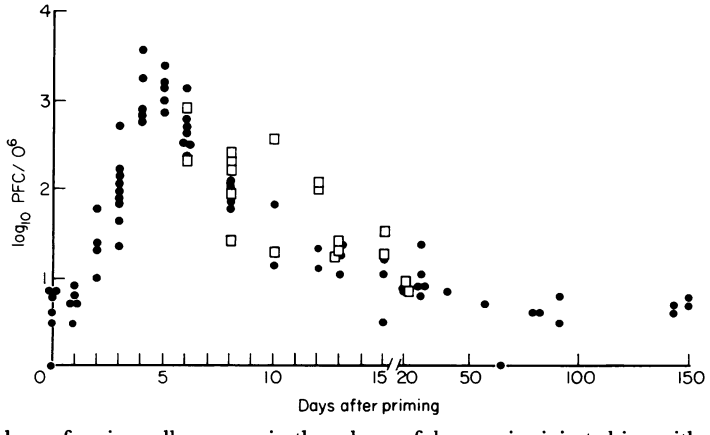

FIG. 1. The plaque-forming cell response in the spleens of donor mice injected i.v. with 5×10^6 sheep erythrocytes. ●, 19S plaque-forming cells; □, 7S plaque-forming cells.

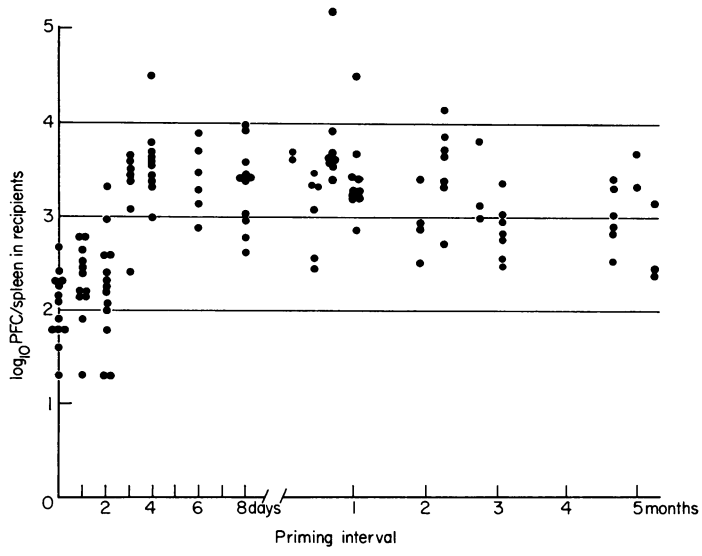

FIG. 2. 19S plaques in the spleens of irradiated recipient mice, assayed 7 days after injecting i.v. 5×10^5 primed donor spleen cells with 2×10^8 sheep erythrocytes. The interval between priming and killing of donor mice is shown on the abscissa.

7 days later. This antigen responsiveness must either be carried by fifty plaque-forming cells, or by 5×10^5 non-plaque-forming cells, or both. To decide this, fifty plaque-formers could be gathered together by micromanipulation and injected into an irradiated recipient, with antigen. Then 5×10^5 spleen cells might be collected from droplets containing no plaques, as described earlier, and this plaque-free population injected into another mouse. It would be expected that the sum of the responses of these two recipients would be roughly equal to the response of a third recipient injected with 5×10^5 randomly selected spleen cells (Fig. 3).

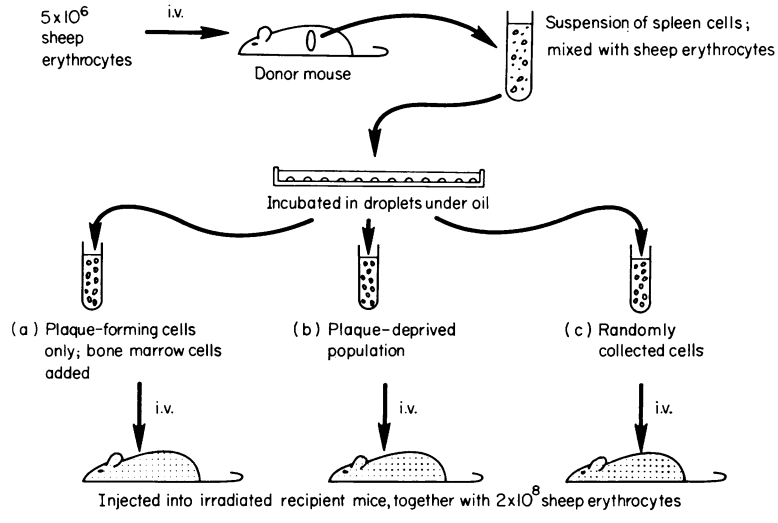

FIG. 3. 'Balance-sheet' experimental design.

The balance-sheet experiments were carried out according to this general plan, with some additional controls. Four irradiated mice were injected in each group of recipients. Cells from the donor animals were collected into 1 ml of medium in six siliconized tubes, held on melting ice. Tube 1 contained the plaque-forming cell equivalent of $2–4\times10^6$ spleen cells. If the donor mouse had 100 plaques/10^6 spleen cells, 200–400 plaque-formers were collected, together with a total of about 4×10^4 'contaminants'. To these spleen cells, 4×10^6 bone marrow cells were added. Tube 2, the contaminant control, contained 4×10^4 random cells treated in the same way as the cells in group (1), and 4×10^6 bone marrow cells. Tube 3, the plaque-free group, contained 2×10^6 spleen cells, collected from droplets containing no plaque-formers. Group 4 was made up of 2×10^6 random cells, incubated and collected in the same way as groups 1–3. Group 5 comprised 2×10^6 cells, randomly collected but not incubated. Sheep erythrocytes (8×10^8) were added to tubes 1–5. Tube 6 was the same as group 5, but without added antigen. One-tenth of each tube (0·1 ml) was then assayed for plaque-forming cells. As expected, plaques were very rarely detected in group 3, confirming the efficiency of the plaque-exclusion process. Between 50 and 100 per cent of the expected number of plaque-forming cells were usually found in group 1: to cover any apparent loss, excess plaque-formers were collected into this tube.

Finally, the contents of each tube were injected into four recipient mice which had been lethally irradiated 1 day before. Seven days after injection, these mice were killed, a dispersed-cell suspension made from their spleens, and a fraction (usually 1 : 20) of this suspension assayed for 19S and 7S plaques.

RESULTS

STANDARDIZATION EXPERIMENTS

An extensive series of experiments was carried out to define the best conditions for demonstrating IgM memory in this test system. The priming dose of sheep erythrocytes injected into donor mice was found to be particularly critical (Sercarz and Byers, 1967). Very high (2×10^9) or very low (5×10^4) doses of sheep erythrocytes gave about ten times less memory (measured at 2, 4, 8, 15 and 29 days after priming) than the optimal number, 5×10^6. That is, irradiated mice injected with 5×10^5 spleen cells from donors primed with high or low doses of sheep erythrocytes produced less plaque-forming cells on challenge than did recipients of spleen cells from donors originally primed with 5×10^6 sheep red cells. Fig. 1 records the plaque-forming cell response in donor mice. Fig. 2 shows how the capacity of 5×10^5 spleen cells to respond to antigenic challenge in recipient mice rises rapidly in the primed donor animals, reaching a peak at about 4 days, and remaining elevated for at least 5 months. Significant numbers of developed (presumably 7S) plaques were present in only a small proportion of irradiated mice. Recipients were killed 7 days after injection, at which time it was found that the peak plaque-forming cell response of the cultured cells was reached, irrespective of the interval between priming and killing of the donor mice.

SPECIFICITY OF MEMORY

This was confirmed by transferring, to irradiated recipients, cells from mice primed with horse erythrocytes. No significant plaque-forming cell response to sheep red cells was observed in the recipient mice.

THE INCREASED NUMBER OF MEMORY CELLS PRODUCED BY IMMUNIZATION

It was necessary to prove that the increased antigen responsiveness of a given number of spleen cells from a primed donor animal reflected an increased proportion of responsive units (memory cells), and not merely an increased potential for proliferation by a constant number of progenitor cells. In dose–response experiments, an approximately linear relationship was found between the number of unprimed donor spleen cells transferred to irradiated mice and the number of plaques per recipient spleen, down to about 5×10^5 transferred donor cells, when levels of plaque-forming cells were not significantly higher than background. By contrast, in similar experiments when spleen cells were transferred from donor mice primed 3, 10 or 40 days before, ten- to thirty-fold less cells were required to give a significant response (Fig. 4). This confirms the findings of Albright and Makinodan (1965), but at the level of the number of antibody-forming cells rather than of serum antibody titres.

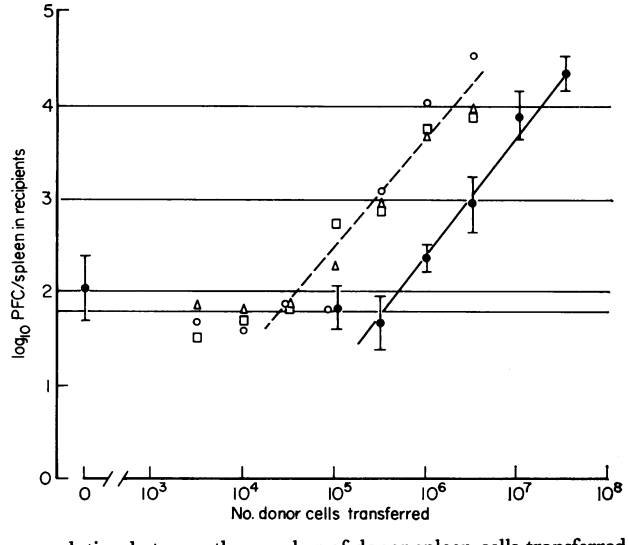

FIG. 4. Dose–response relation between the number of donor spleen cells transferred (by i.v. injection, together with 2×10^8 sheep erythrocytes), and the number of plaque-forming cells 7 days later in the spleens of irradiated recipient mice. Ninety-five per cent confidence limits of the mean responses in one group are shown; they were of similar magnitude in the other groups, but have been omitted for clarity. Interval between priming and killing donor mice was 0 (●), 3 (△), 10 (□) or 40 (○) days.

BALANCE-SHEET EXPERIMENTS

Table 1 records the results of a typical balance-sheet experiment (see 'Materials and methods') in which the donor spleen cells were taken from a mouse 29 days after priming. The spleens of irradiated mice in group 1, which received donor plaque-forming cells and bone-marrow cells, contained numbers of plaque-forming cells which were not significantly higher than background. The low counts in group 2 mice confirmed that the number of contaminant cells injected into group 1 animals together with the plaque-formers was not enough to give a response. On the other hand, group 3 mice, which received the plaque-free population, responded just as vigorously as group 4 recipients which were injected with 5×10^5 random incubated cells. Group 5 was included to check that memory cells were not damaged by the incubation or collection. Group 6 mice

TABLE 1

RESULTS OF 'BALANCE-SHEET' EXPERIMENT CARRIED OUT 29 DAYS AFTER PRIMING THE DONOR MOUSE (DONOR SPLEEN HAD 24 19S PFC/10^6 CELLS)

Groups	Total cells injected into each recipient mouse		PFC/spleen in recipient mice
	Spleen cells	Bone marrow cells	
(1) PFC*	5×10^3 approx.	10^6	40 220 100 100
(2) Contaminant control	10^4	10^6	200 200 180 40
(3) Non-PFC	5×10^5	–	2200 4280 5600 6560
(4) Whole population	5×10^5	–	1640 2640 1880 1760
(5) Whole population, not incubated	5×10^5	–	740 32000 2680 1960
(6) Whole population, no antigen	5×10^5	–	<20 40 40 280

* PFC = Plaque-forming cells.

received spleen cells but not antigen: apparently, in the absence of re-stimulation with antigen, little or no further proliferation of antibody-forming cells occurs in the host.

A balance-sheet experiment was carried out on the spleen cells from an unprimed donor mouse in the same way except that larger numbers of cells had to be transferred to produce significant responses in recipient mice (Tables 2 and 3). None of the antigen responsiveness seemed to reside in the background plaque-forming cells. To test this further, thirteen recipient mice were each injected with from ten to forty background plaque-formers from five unprimed donor mice. Only one of these recipient mice gave a significant response; its spleen contained about 4700 plaque-forming cells, some seventy of which produced very large plaques (from 1 to 3 mm in diameter after 1 hour's incubation), perhaps indicating abnormal division of antibody-forming cells in an inadequately irradiated animal.

In all ten of the balance-sheet experiments (Fig. 5, Table 3), no memory was transferred by the plaque-forming cell (group 1), population. At 8, 12, 29 and 79 days after priming, all the memory capable of being expressed in this adoptive transfer system was carried by non-plaque-forming cells. Four experiments were done 3 days after priming donor mice, because this was the time at which the increase in numbers of plaque-forming cells and the rise in memory cells were most closely parallel. Memory was less marked at this time than at later points, but significant memory was demonstrated in the plaque-free population in three out of four experiments, and none was found associated with the plaque-forming cells themselves. One experiment where the priming interval was 2 days gave very poor memory, in spite of the fact that 2×10^6 cells were transferred to each recipient in groups 4, 5 and 6. The process of incubation and collection may have damaged the memory cells in this experiment (Table 3).

TABLE 2

RESULTS OF A 'BALANCE-SHEET' EXPERIMENT CARRIED OUT USING SPLEEN CELLS FROM AN UNPRIMED MOUSE (DONOR SPLEEN HAD APPROXIMATELY 1 19S PFC/10^6 CELLS)

Groups	Total cells injected into each recipient mouse		PFC/spleen in recipient mice
	Spleen cells	Bone marrow	
(1) PFC*	2×10^3 approx.	10^6	140 40 160 600
(2) Contaminant control	2×10^3	10^6	60 300 140 120
(3) Non-PFC	5×10^6	–	36800 8000 4160 9840
(4) Whole population	5×10^6	–	2680 2360 4560 5200
(5) Whole population not incubated	5×10^6	–	5480 4480 6400 5680
(6) Whole population, no antigen	5×10^6	–	<20 80 160 300

* PFC = Plaque-forming cells.

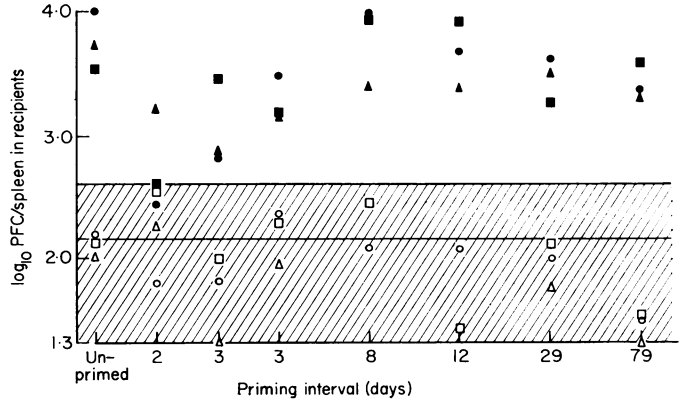

FIG. 5. Results of eight of the ten 'balance-sheet' experiments. The geometric mean number of plaque-forming cells per spleen in each group of recipients has been plotted. Group 6 was not included in the experiments done at 8 and 12 days. The horizontal line and shaded area represent the mean and range of plaque-forming cells per spleen found 7 days after injecting twenty-three irradiated mice with 10^6 bone marrow cells and 2×10^8 sheep erythrocytes (full data on the number of cells transferred to each recipient group is given in Table 3). ○, PFC alone (1); □, contaminant control (2), ●, no plaques (3); ■, all, incubated (4); ▲, all, not incubated (5); △, minus Ag control.

TABLE 3

SUMMARY OF ALL BALANCE-SHEET EXPERIMENTS (THE GEOMETRIC MEAN OF THE PFC/SPLEEN IN SURVIVING RECIPIENTS (EXPRESSED AS $\log_{10}$) IS RECORDED FOR EACH GROUP)

	Interval between priming and killing of donors (days)									
	Unprimed	2	3	3	3	3	8	12	29	79
19S PFC/10^6 in donor spleen	1	13	86	67	61	69	120	13	24	4
PFC injected into each recipient in group 1	8	26	88	67	62	70	60	19	24	9
Total spleen cells injected per mouse in groups 4, 5 and 6	5×10^6	2×10^6	10^6	10^6	10^6	10^6	$0{\cdot}5\times10^6$	10^6	$0{\cdot}5\times10^6$	10^6
Total spleen cells injected per mouse in group 3	5×10^6	2×10^6	$0{\cdot}5\times10^6$	$0{\cdot}75\times10^6$	$0{\cdot}75\times10^6$	10^6	$0{\cdot}4\times10^6$	$0{\cdot}5\times10^6$	$0{\cdot}5\times10^6$	10^6
Groups										
1	2·18	1·78	2·38	1·80	2·49	2·35	2·08	2·06	1·99	1·50
2	2·12	2·53	2·11	1·99	2·47	2·29	2·46	1·42	2·12	1·53
3	4·02	2·43	2·88	2·82	3·18	3·50	3·98	3·69	3·64	3·38
4	3·55	2·62	3·59	3·48	3·86	3·18	3·96	3·94	3·29	3·62
5	3·74	3·20	3·40	2·86	2·92	3·15	3·40	3·38	3·52	3·34
6	1·97	2·23	1·90	1·30	2·12	1·93	*ND	ND	1·74	1·30

* ND = Not done.

DISCUSSION

The main aim of this work was to decide whether or not antibody-forming cells carry immunological memory. That is, do antibody-forming cells themselves, when exposed to specific antigen, generate increased numbers of further antibody-formers, either by proliferating or by causing the proliferation of other cells? Direct evidence of this point is difficult to obtain with whole-animal studies, so an adoptive transfer system was used. This had two advantages: (a) the memory carried by spleen cells from donor mice could be assayed without interference from free antibody or other unknown and uncontrollable factors in the primed animals; (b) the antibody-forming cell content of the populations whose memory was assayed could be regulated experimentally. A modification (Cunningham, 1968) of the haemolytic plaque technique was used to identify antibody-plaque-forming cells, because it was essential that the cells should be undamaged and readily recovered after a period of incubation. Spleen cell populations from donor mice were then separated, by micromanipulation, into plaque-forming cells and non-plaque-formers. These two fractions were injected into different irradiated recipient mice, and the response to challenge of these mice was compared with the response of further irradiated animals restored with unfractionated spleen cells.

Only IgM (direct) plaques were found consistently in recipient mice, irrespective of the priming dose of antigen administered to donor mice. The reason for this is unknown. It contrasts with Möller's (1968) demonstration of numerous 7S plaque-forming cells in the spleens of irradiated mice, which had been restored with relatively large numbers of spleen cells from hyperimmunized donors.

IgM memory was found to be long lasting. The amount of antigen responsiveness carried by 5×10^5 cells from primed donor mice rose to about thirty times that of normal spleen cells by 4 days after priming, and this average increment was maintained (with no more than a hint of decline) for at least 5 months (Fig. 2), although individual responses in irradiated recipients varied greatly. Again, this contrasts with the transience of the IgM memory which has been encountered in whole animal studies (Wigzell, 1966; Sercarz and Byers, 1967). In the course of a secondary response in normal animals, IgM production is probably rapidly aborted by the early synthesis of large amounts of IgG antibody (Möller and Wigzell, 1965).

Celada (1967) described a decay in memory when primed cells were transferred to an inert recipient, then left for varying intervals before challenge. In the present experiments the cells were challenged at the time of transfer, having been left for different times in the donor, when, in agreement with the findings of Nettesheim, Makinodan and Williams (1967), no significant decay was observed. It may be that the intact donor animal provides a better environment for the survival of memory cells than does the recipient to which they are eventually transferred. Alternatively, or in addition, memory cells may be continuously generated in the primed animals, perhaps through the action of persisting antigen.

Dose–response experiments (Fig. 4) showed that the increase in antigen-responsiveness of spleen cells from primed mice was caused by the appearance of an increased proportion of antigen-reactive cells (Albright and Makinodan, 1965). The generation of these cells is probably the fundamental event in the development of immunological memory. However, administering large doses of antigen to donor mice produced sub-optimal memory. This finding suggests that, on repeated exposure to antigen, cells may lose the property of memory, possibly by a process of exhaustive differentiation, as has been proposed for the antibody-forming cell series (Albright and Makinodan, 1965; Sterzl, 1966).

At all times tested, the IgM memory carried by spleen cells was a property of cells which were not releasing detectable antibody. The results were clear-cut, but some possible limits to the general applicability of the conclusions must be admitted. It is not excluded that memory cells do produce extremely small amounts of antibody, although the technique used to identify antibody-forming cells in the present study was capable of detecting lysis of ten to twenty erythrocytes. Furthermore, only the spleens of irradiated recipients were examined, a plaque assay on these being a much more sensitive test, and a more direct measure of cellular events than an antibody titration of the whole animal's serum. It could be argued that the proportion of cells lodging in the spleen is not representative of the total injected population, and it was to overcome this objection that the 'balance-sheet' experimental design was used. The observation that transferred plaque-forming cells alone do not give rise to a response in the spleens of challenged recipient mice does not in itself prove that memory cells are not plaque-formers. However, the complementary demonstration, that a population completely deprived of detectable plaque-forming cells is just as capable of producing a response in recipient spleens as is an unfractionated population, seems greatly to strengthen the conclusion that plaque-forming cells and memory cells are distinct.

Memory must be a property either of the plaque-forming *line* or of a separate line of cells. If the first alternative is true, memory must be carried by 'pro-plaque-forming' cells, cells which will later produce antibody or antibody-forming progeny, or by 'post-antibody-forming' cells, cells which have finished releasing antibody, or by the antibody-forming cells themselves (Fig. 6). The present demonstration that plaque-forming cells do not

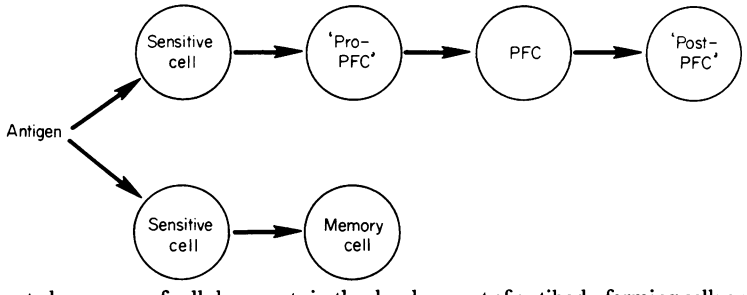

FIG. 6. Suggested sequence of cellular events in the development of antibody-forming cells and immunological memory.

carry memory also makes the possibility that post-plaque-formers are the memory cells less attractive; one would have expected transferred plaque-forming cells to turn into memory cells in recipient mice, and so to confer antigen-responsiveness on them. On the other hand, if pro-plaque-formers are the memory cells, then vigorous memory should develop before the plaque response in donor mice. This was not observed in the present study. Sercarz and Byers (1967), using the sensitive Net Excess Secondary Response index of Nossal, Austin and Ada (1965), were able to demonstrate some memory at 1 day, about the time when the plaque-forming cell response begins. However, they also found that memory did not reach maximum levels before the peak of the plaque-forming cell response.

The experiments described here favour the idea that memory is carried by a line of cells distinct from the antibody-forming cells. This would explain situations where memory develops without antibody formation (Nossal *et al.*, 1965). Also it is in agreement with the

recent suggestions of Mitchell and Miller (1968) that, whereas antibody formation is carried out by a line of cells arising from bone marrow, specific antigen responsiveness is a property of a separate line of thymus-derived cells. The fact that memory develops so rapidly in mice injected with sheep erythrocytes suggests that antigen stimulates extensive proliferation of antigen-responsive or memory cells. The IgM plaque-formers themselves, although they all arise by mitotic division (Szenberg and Cunningham, 1968), may have a relatively limited capacity for proliferation.

ACKNOWLEDGMENTS

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