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Studies on the Cellular Basis of IgM Immunological Memory

THE INDUCTION OF ANTIBODY FORMATION IN BONE MARROW CELLS BY PRIMED SPLEEN CELLS

A. J. CUNNINGHAM

Wallaceville Animal Research Centre, Department of Agriculture, Private Bag, Wellington, New Zealand

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Summary. Immunological memory and antibody formation appear to be properties of different cell lines. This follows from a demonstration that cells from spleens of mice primed with sheep erythrocytes are capable of inducing normally unresponsive bone marrow cells to produce specific antibody.

Primed spleen cells, taken from CBA or CBAT6T6 mice, were cultured for 5 or 6 days with an excess of bone marrow cells of the opposite chromosome type, and with antigen, in lethally irradiated, histocompatible mice. The tissue of origin of antibody-plaque-forming cells from the spleens of the irradiated mice was then identified from chromosome spreads of individual plaque-forming cells. After 5 days of culture, equal numbers of spleen and bone marrow cells were found producing antibody. At 6 days nearly all of the plaque-forming cells were from the bone marrow. Plaque-forming cells of bone marrow origin were also identified, using an immunogenetic technique, when semi-allogeneic mixtures of spleen and bone marrow cells were cultured together.

INTRODUCTION

Immunological memory must be carried either by the antibody-forming cell line, or by a separate line of cells. The first alternative has been widely promulgated, for example in the 'X–Y–Z' scheme (Sercarz and Coons, 1962; Sercarz and Byers, 1967), in which 'X' are precursor cells, the 'Y' cells carry memory, and their progeny, 'Z', produce antibody. The second possibility has not received much attention: it appears to imply transfer of specific information from memory cells to the cells which are induced to form antibody.

In an earlier paper (Cunningham, 1969), an attempt was made to decide between these alternatives by direct experiment. Relatively pure populations of antibody-plaque-forming cells (PFC) were tested for their ability to react to antigen in irradiated recipient mice. It was shown that memory was not carried by PFC, and probably not by precursors nor progeny of the antibody-formers. IgM immunological memory to sheep erythrocytes therefore appeared to be a property of a separate line of cells.

The present paper strengthens this conclusion by demonstrating an apparent induction of antibody formation in one cell line by memory cells from another. The experiments are based on two observations by others. First, the finding of Radovich, Hemingsen and Talmage (1968) that the PFC response of lethally irradiated mice injected with primed

spleen cells, is enhanced by addition of a large number of bone marrow cells to the inoculum. Secondly, the demonstration by Miller and his colleagues (Miller and Mitchell, 1968; Mitchell and Miller, 1968; Nossal, Cunningham, Mitchell and Miller, 1968), that the response of mice to sheep erythrocyte antigen may depend, in some experimental situations, on an interaction between thymus or thoracic duct cells, which recognize antigen, and cells from bone marrow which are induced to produce antibody.

MATERIALS AND METHODS

Mice

CBAT6T6 mice were obtained, in 1967, from the Medical School, University of Otago, New Zealand, and have been maintained since then by brother–sister mating in our own colony. The CBA/p strain, histocompatible with CBAT6T6 but with a distinctive fawn-coloured coat, is said to have originated as a mutation in the CBA colony at the University of Cambridge, England (Snell, Staats, Lyon, Dunn, Gruenberg, Hertwig and Heston, 1960). The nucleus of our colony of CBA/p mice was also obtained from the University of Otago. C57BL mice came from Dr R. E. Munford, Massey University, Palmerston North. The (CBAT6T6 × C57BL) F_1 hybrid mice were bred in our own colony.

Animals 8–14 weeks old of either sex were used. No problems were encountered in distinguishing between the T6 chromosomes and the Y chromosomes of male mice.

Irradiation

Mice received 900 rads of ^{60}Co whole body γ-irradiation, at a dose rate of 15 rads/min.

Priming donor mice

An intravenous injection of 5×10^6 sheep red cells was given 4 days before killing. This relatively small dose of antigen has previously been found to provide optimal priming for the adoptive transfer system (Cunningham, 1969). In three experiments, the interval between priming and killing donor mice was 10 or 11 days.

Preparing cell suspensions

The medium used throughout was Hanks's balanced salt solution with 5 per cent foetal calf serum. Spleen cell suspensions were prepared by rubbing spleens on stainless steel wire mesh under medium. Bone marrow cells were expelled from femurs and tibiae of normal mice using a syringe and 26-gauge needle. Clumps of cells were removed from cell suspensions by centrifugation at 100–200 ***g*** for a few seconds. The dispersed cells were then collected by centrifugation at 400 ***g*** for 5 minutes, then resuspended to the appropriate concentrations in fresh medium.

Culturing cells in irradiated mice

One day after irradiation, recipient mice were injected intravenously with 10^6 spleen cells from primed donor mice, together with 2×10^8 sheep erythrocytes and $10–20 \times 10^6$ normal bone marrow cells. Spleen and bone marrow cells were always of opposite chromosome type, and in most experiments the bone marrow cells were of the same chromosome type as the irradiated host. For example, CBAT6T6 bone marrow cells plus CBA/p (non-T6) primed spleen cells were cultured together in CBAT6T6 irradiated hosts. CBA/p hosts received CBAT6T6 primed spleen and CBA/p bone marrow.

Controls included mice injected with cell mixtures lacking one or more of the three components: primed spleen, bone marrow or sheep erythrocytes.

A series of standardisation experiments was initially carried out using different numbers of bone marrow and primed spleen cells, and, in some cases, different priming doses of antigen to the mice donating the spleen cells.

Assays for PFC

A sensitive modification of the haemolytic plaque technique was used (Cunningham and Szenberg, 1968).

Selection of single PFC for chromosome analysis

Irradiated mice were killed 5 or 6 days after the injection of donor cells and antigen. 'Colcemid' (4 μg/g body weight) was administered intraperitoneally 1½–2 hours before killing. Dispersed spleen cell suspensions were made and incubated at 37° together with indicator erythrocytes and complement, as stable monolayers in microdroplets of medium under paraffin oil (Cunningham, 1969). The concentration of spleen cells in these droplets was not allowed to exceed 10^6 cells/ml to ensure that, in a high proportion of plaques, the active cell could be recognized as the only nucleated cell near the centre.

After 20 minutes incubation, microdrops were scanned at 35- and 100-fold magnification, for plaques containing a central cell in metaphase. The proportion of PFC which were in metaphase is not accurately known, but it was small—around 1–2 per cent. These cells were recognized by their large size, regular round shape, and vaguely ribbed appearance (Nossal *et al.*, 1968).

Preparing chromosome spreads from individual PFC

The method used was an improvement of the 'micro-Ford' technique described earlier (Nossal *et al.*, 1968). Using a micropipette mounted on an improvised micromanipulator, a PFC thought to be in metaphase was transferred to a large drop (about 0·1 ml) of 1 per cent sodium citrate on a clean slide. This drop was held in place by a ring of paraffin wax–Vaseline (50 : 50) mixture. One or more PFC were collected over a period of about 45 minutes. While not in use, the slide supporting the citrate solution was held in a moist chamber to prevent evaporation.

Next, about 0·2 ml of 10 per cent Clarke's fixative (3 parts ethanol to 1 part glacial acetic acid, diluted 1 : 10 in 1 per cent citrate solution), was very carefully pipetted into the drop of citrate. This fixed the cells firmly to the slide. In those cells that would later produce good spreads, the chromosomes were clearly displayed at this stage as dark patches evenly spread throughout the cell. The 10 per cent Clarke's solution was then replaced by two changes of undiluted fixative. This was left over the cells for 10 minutes. All operations up to this point were carried out with the slide on the microscope stage and observed at $\times 35$ or $\times 100$ magnification.

The last step, spreading the cells, was the most critical. It was modified several times during the course of these experiments. At first, cells were spread by breaking the paraffin–vaseline ring, and allowing the fixative to evaporate rapidly. Later it was found that the quality of spreads could be improved if the Clarke's fixative was replaced by 40 per cent acetic acid, which was then heated, and quickly sucked away from the cells with filter paper (suggested by Dr M. A. S. Moore). This procedure appeared to rupture the cells more violently, distributing the chromosomes over a wider area. However, a proportion of

cells was lost using this technique. Recently, this loss has been avoided by removing the paraffin–Vaseline ring, evaporating the Clarke's fixative almost to dryness, then adding 40 per cent acetic acid, heating, and quickly sucking it off the slide.

The main difference between this procedure and the 'micro-Ford' technique described originally (Nossal *et al.*, 1968), is that cells are now fixed before final spreading, and the spreading step is more controlled: preparations are now of comparable quality to those produced by standard macro-techniques (Fig. 1).

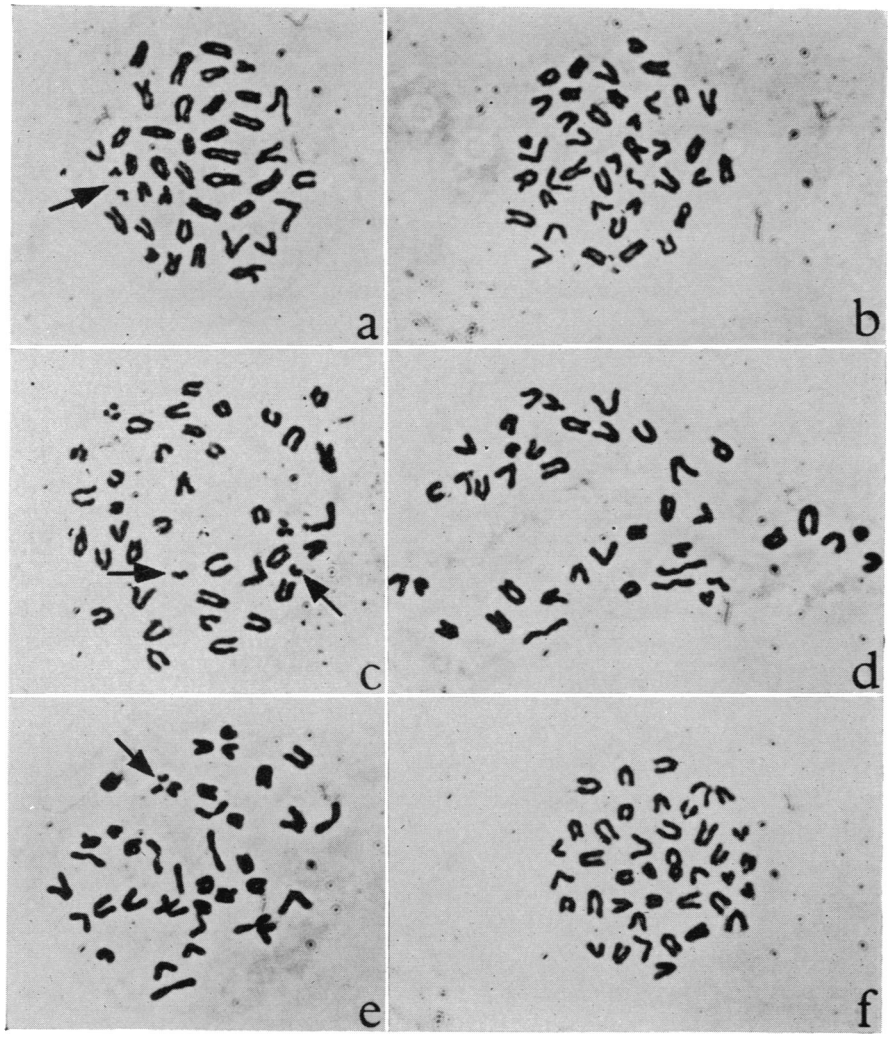

FIG. 1. Six chromosome spreads, prepared from individual PFC. (a), (c) and (e) are CBAT6T6 cells (with the T6T6 marker chromosomes arrowed), while (b), (d) and (f) are CBA/p (non-T6) cells. (a), (b) and (c) were taken from irradiated CBAT6 mice injected with primed spleen cells from a CBA/p donor and with bone marrow from a CBAT6T6 animal. (a) and (c) are, therefore, of bone marrow origin, while (b) came from the injected spleen cells. (d) was taken from an irradiated CBA/p mouse which had received CBAT6T6 primed spleen and CBA/p bone marrow; it is, therefore, a PFC of bone marrow type. (e) and (f) are PFC of splenic origin from control experiments.

Controls for single-cell chromosome experiments

Mistakes seemed to be possible at two stages in the technique described above: (a) the wrong cell might have been classed as a plaque-former; and (b) in the case of very poor spreads, the final chromosome preparation might have been misread. The importance of these two sources of error was estimated in reconstruction experiments where PFC of known chromosome type were picked out of a mixture containing an excess of cells of the opposite type.

A cell suspension was prepared from the regenerating spleen of an irradiated CBAT6T6 mouse 5 or 6 days after it had been injected with 10^7 CBAT6T6 bone marrow cells. This suspension contained a large number of cells in metaphase, all carrying the T6T6 marker chromosomes. It contained very few PFC (usually less than 200). A second cell suspension was now made from the spleen of a non-irradiated CBA/p mouse 4 days after it was injected with 2×10^8 sheep erythrocytes. This contained fewer cells in metaphase than the first suspension, but vastly more PFC (around 200,000). A portion of the second suspension was mixed with all of the first, to give a mixture containing a large number of PFC, virtually all of CBA/p type, mixed with a lot of other cells in metaphase, mostly of CBA-T6T6 type. Most mistakes, either in picking out PFC or in reading the spreads, would have shown up as cells of T6T6 chromosome type.

In each control experiment, two kinds of mixture were made, one with T6T6 PFC amongst an excess of non-T6 cells in metaphase, and the other with the reverse combination. Cells were incubated at the same concentration as in test experiments. PFC were selected and spread from both mixtures, and read 'blind', that is, with the origin of the cells concealed from the observer.

Semi-allogeneic mixtures of spleen and bone marrow cells

In a series of six experiments, irradiated (CBA$\times$C57BL) F_1 hybrid mice were injected intravenously with 2×10^8 sheep erythrocytes, 10^6 spleen cells from a primed F_1 mouse, and $10–20\times10^6$ parental type bone marrow cells taken from either CBA or C57BL mice. The recipients were killed 5, 6, 7 or 10 days after injection. The tissue of origin of PFC was then identified using cytotoxic antisera able to destroy selectively CBA or C57BL cells (Harris, Harris and Ogburn, 1967).

Cytotoxic sera were prepared against C57BL mice by injecting CBA mice on two or three occasions with approximately 10^7 C57BL or F_1 hybrid spleen cells. Anti-CBA sera were prepared in a similar way. Sera were inactivated and absorbed with sheep erythrocytes before use. Dispersed spleen cells from mice in which semi-allogeneic cell mixtures had been cultured, were incubated for 20 minutes at 37° in medium containing 15 per cent cytotoxic or normal F_1 serum, and 15 per cent guinea-pig serum as a source of complement. Sheep erythrocytes were then added, and the whole mixture was assayed for PFC in slide chambers.

RESULTS

NUMBERS OF PFC IN RESTORED MICE

The spleens of mice which had been irradiated then injected with 10^6 primed spleen cells and 2×10^8 sheep erythrocytes contained between 1500 and 15,000 direct (19S) PFC when examined 5–8 days later. This number was enhanced from one- to five-fold in

different experiments by the simultaneous injection of $10–20 \times 10^6$ histocompatible bone marrow cells. Control mice, receiving sheep erythrocytes alone or erythrocytes plus bone marrow, seldom had more than 600 PFC in their spleens. The results of one of a series of standardization experiments are shown in Table 1.

TABLE 1

NUMBERS OF PFC IN THE SPLEENS OF LETHALLY IRRADIATED CBAT6T6 MICE 6 DAYS AFTER INJECTING THEM WITH THE CELL POPULATIONS LISTED. FIVE MICE WERE USED IN EACH GROUP

Group	Cells injected into irradiated mice			Mean No. PFC/spleen	Standard deviation
	10^6 primed spleen	10^7 normal bone marrow	2×10^8 sheep RBCS		
1	−	−	+	76	30
2	−	+	+	384	214
3	+	+	−	248	70
4	+	−	+	6036	3740
5	+	+	+	11752	3160

SINGLE-CELL CHROMOSOME ANALYSIS

The best yield of PFC in metaphase was obtained on days 5 and 6 after injection when numbers of PFC averaged about 10,000–20,000 per spleen. Table 2 shows that of forty-seven PFC studied in such mice, seventeen came from primed spleen and thirty from bone marrow cells. Fig. 1 illustrates some of the spreads obtained. PFC of bone marrow type were found whether the bone marrow cells were of the same kind as those of the irradiated host, or of the opposite kind.

TABLE 2

THE RESULTS OF CHROMOSOME ANALYSES ON SINGLE PFC ISOLATED FROM MIXTURES OF PRIMED SPLEEN AND BONE MARROW CELLS CULTURED FOR 5 OR 6 DAYS IN LETHALLY IRRADIATED SYNGENEIC HOSTS

Days of culture	No. of PFC found with the indicated chromosome type					
	'Definite' diagnosis		'Probable' diagnosis		Total	
	Primed spleen	Bone marrow	Primed spleen	Bone marrow	Primed spleen	Bone marrow
5	6	9	7	4	13	13
6	0	5	1	6	1	11
6*	1	4	2	2	3	6

* In these experiments, the bone marrow cells and the lethally irradiated host were of opposite chromosome type. In all others, bone marrow cells were of the same type as the host mouse.

TABLE 3

CONTROLS FOR SINGLE-CELL CHROMOSOME EXPERIMENTS

'Definite' diagnosis		'Probable' diagnosis		Total	
Spleen type (correct)	Bone marrow type (mistake)	Spleen type (correct)	Bone marrow type (mistake)	Correct	Mistakes
16	0	19	2	37	2

Virtually all PFC were of spleen type, and these cells were picked out and identified 'blind' from a mixture in which most cells in metaphase came from bone marrow.

CONTROLS FOR SINGLE-CELL CHROMOSOME EXPERIMENTS

In these experiments, PFC of one chromosome type were picked out of a mixture in which most cells in metaphase were of opposite type (see 'Materials and methods'). No mistakes were made in selecting or reading the sixteen good spreads (Table 3), while two mistakes occurred in scoring twenty-one poorer spreads. Preparations made from the cell mixtures confirmed that metaphase cells of bone marrow type were, on average, three times as numerous as cells of the PFC (spleen) type. In other words, of any four mistakes, an average of three would have been detected as cells of unexpected chromosome type.

SEMI-ALLOGENEIC CELL COMBINATIONS

In five of the six experiments, the presence of excess parental bone marrow did not enhance the PFC response of F_1 primed spleen cells. At 5, 6 and 10 days there was no good evidence for PFC of bone marrow origin, although the number of plaques examined was small. However, in all of three experiments done at 7 days, PFC of bone marrow type were clearly present. Table 4 shows that the anti-CBA serum effectively destroyed nearly all plaques in control cell mixtures containing only F_1 (group 1) or CBA (group 2) PFC, while it had little effect on a cell suspension in which all PFC were known to be of C57BL type

TABLE 4

IMMUNOGENETIC ANALYSIS OF THE ORIGIN OF PFC IN SYNGENEIC AND SEMI-ALLOGENEIC MIXTURES OF PRIMED SPLEEN AND BONE MARROW CELLS, CULTURED IN LETHALLY IRRADIATED (CBA × C57BL) F_1 HOSTS

Group	Cell mixture cultured in irradiated F_1 mouse	Plaques after incubation with normal F_1 serum	Percentage reduction after incubation with:	
			Anti-CBA	Anti-C57BL
1	F_1 primed spleen	74	93	97
2	CBA primed spleen + CBA bone marrow	111	94	23
3	C57BL primed spleen + C57BL bone marrow	28	9	100
4	F_1 primed spleen + CBA bone marrow	148	92	42
5	F_1 primed spleen + C57BL bone marrow	41	12	100

(group 3). A mixture of F_1 primed spleen and C57BL bone marrow (group 5) was virtually unaffected by the same serum, strongly suggesting that most of the PFC in this mouse were of bone marrow origin. Similarly, the anti-C57BL serum almost entirely prevented plaque formation in control mixtures where all PFC were of C57BL type (groups 1 and 3), while it had little effect on known CBA PFC (group 2). Less than half the PFC in a mixture of F_1 primed spleen and CBA bone marrow were destroyed by this serum (group 4), indicating again that most PFC were derived from bone marrow cells, although it is probable that a proportion of F_1 PFC were also present.

DISCUSSION

When a mouse is injected with sheep erythrocytes, its spleen generates a greatly increased number of cells capable of reacting specifically to a second contact with the same antigen (Cunningham, 1969). These memory cells are not IgM PFC, and are probably not precursors or descendants of the PFC. The present paper strengthens the conclusion

that memory cells and antibody-formers belong to different cell lines by demonstrating that when primed spleen cells are cultured with antigen and an excess of bone marrow cells in a lethally irradiated mouse, bone marrow cells are induced to produce antibody. This induction takes place over not more than 5 or 6 days, a period in which bone cells cultured with antigen alone give virtually no response.

That the presence of bone marrow cells will enhance the response of cultured primed spleen cells is in agreement with the findings of Radovich *et al.* (1968). The enhancing effect observed here was relatively small, probably because a different priming dose of antigen was used: an optimal priming dose may leave less room for enhancement of a response by bone marrow. Radovich *et al.* (1968) suggested that enhancement was a relatively non-specific effect, caused mainly by increased overall numbers of cells in the spleens of mice which received bone marrow. This explanation may well be partly correct, but also it appears that bone marrow contributes precursor cells which are capable of being induced to form antibody, by memory cells from primed spleen. Because of the availability of two effectively syngeneic lines of CBA mice, one of which has two distinctive marker chromosomes, it was possible to demonstrate PFC of bone marrow origin directly, by analysing the chromosome pattern of single antibody-forming cells.

The method for examining metaphase chromosomes in single antibody-forming cells was originally devised by Nossal *et al.* (1968), and has now been improved to yield spreads as good as those usually obtained with conventional macro-methods. It is tedious to use when the proportion of cells forming antibody is less than 0·1 per cent and the proportion of these PFC apparently in metaphase is of the order of 1 per cent. Under these conditions, an average day's work yielded two to four readable spreads.

Although the total number of cells examined was small, it was clear that the number of bone marrow cells producing antibody was disproportionately high. When 10^7 or more bone marrow cells were cultured with antigen in irradiated hosts, a very small response was obtained (about 400 PFC). The addition of 10^6 primed spleen cells to the bone marrow cells boosted this response about thirty-fold. If this total response were merely an additive effect of the separate responses of spleen and bone marrow, then about one cell in thirty would by chance have been of bone marrow type. The fact that of forty-seven cells examined, thirty originated from the bone marrow was clearly inconsistent with this hypothesis, and the process by which primed spleen cells increased the antibody response of bone marrow cells has been called 'induction' in this discussion.

The validity of results obtained with the single cell technique was established by control experiments in which PFC of known chromosome type were successfully isolated (in 95 per cent of trials) from artificial mixtures containing a majority of metaphase cells of the opposite type. The possibility that cells of the lethally-irradiated host were restored to competence was eliminated in experiments where antibody formation was induced by primed spleen cells with the karyotype of the irradiated host in bone marrow cells of the opposite kind (Table 2).

These results were confirmed in a limited series of experiments using an immunogenetic technique described by Harris *et al.* (1967). The response of spleen cells from primed (CBA × C57BL) F_1 hybrid mice was, if anything, depressed by the simultaneous injection of excess parental bone marrow cells. This lack of enhancement in semi-allogeneic combinations was also noted by Mitchell and Miller (1968). Nevertheless, evidence was obtained for the presence of PFC of bone marrow origin in such mixtures by treating them with cytotoxic antisera capable of destroying F_1 but not parental cells. This method is

much more convenient than the single cell chromosome technique, but it can only be applied to the analysis of mixtures of antigenically different cells which are able to exist side by side for the duration of an experiment.

The involvement of bone marrow cells in antibody production demands some explanation, since in an immunized intact animal, PFC are rare in the bone marrow itself (Friedman, 1964). It seems probable that these cells are immunologically inert in their normal environment, but in the new surroundings provided by an irradiated spleen, some of them may be induced to differentiate into what Mitchell and Miller (1968) have called 'antibody-forming cell precursors'. A spleen presumably contains both antibody-forming cell precursors and antigen-reactive cells, which explains why half of the PFC found at 5 days after injection in the present experiments (Table 2) were of spleen chromosome type, while at 6 days, they were mostly derived from marrow: the inference is that the maturation of precursor cells from normal bone marrow cells, followed by their induction for antibody formation, takes 5–6 days.

Little can be said about the nature of the interaction between memory cells and bone marrow cells, beyond the fact that it requires a specific antigenic stimulus to initiate it. It is probably of the same kind as the interactions between thymus or thoracic duct lymphocytes and bone marrow cells which have been described by Claman, Chaperon and Triplett (1966) and by Miller and his colleagues (Miller and Mitchell, 1968; Mitchell and Miller, 1968; Nossal *et al.*, 1968). Because memory is specific, the 'memory cells' must be committed to react with the specific antigen. On the other hand, the range of potential reactivity of individual bone marrow cells is unknown. The bone marrow cells would not produce antibody if cultured alone with antigen, but are somehow induced to do so by the presence of cells from primed spleen. Four possible kinds of interaction are shown in Fig. 2. First, the effect may be entirely non-specific, for example, the production of some factor

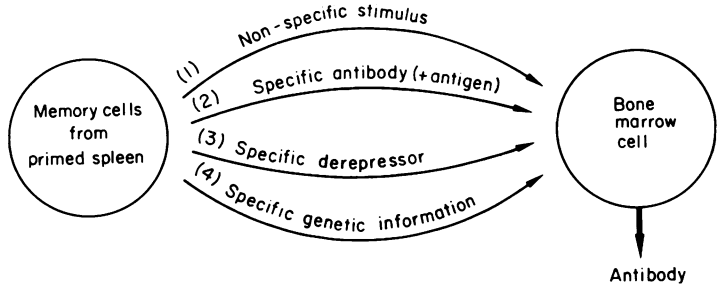

FIG. 2. Four hypothetical types of interaction which might take place between memory cells and bone marrow cells. They are arranged in order of increasing specificity of the 'information' which is assumed to be transferred between the cells.

by the spleen cells which accelerates the growth of immunocompetent cells in bone marrow. This seems most unlikely, otherwise priming the spleen cell donors should have no effect, and it is known that primed spleen and bone marrow generate many more PFC than unprimed spleen and bone marrow (Radovich *et al.*, 1968; Cunningham, unpublished). Secondly, cells from primed spleen may release an antibody that combines with antigen and increases its immunogenicity for immunocompetent bone marrow cells. There is some precedent for this in the relatively small enhancing effect obtained by complexing IgM antibody with sheep erythrocytes (Henry and Jerne, 1968). However, this explanation

also seems unlikely because cells producing IgM antibody do not carry memory, and because numbers of memory cells remain high for some months after antibody production has fallen to a low level (Cunningham, 1969). A third possibility is that the memory cells transfer a specific stimulus (derepressor?) to the bone marrow cells which causes the latter to synthesize antibody coded for by their own genomes. Finally, the genetic information for synthesizing the antibody may come from the primed spleen cells. This last model predicts that characteristics of the primed spleen cell line should appear on the antibody produced by the bone marrow cells, for example, as allotypic markers.

Whether there is any qualitative difference between the cellular events of primary and subsequent immune responses in unknown. This study has highlighted the fact that the number of antigen-reactive cells in a spleen is considerably increased by priming. Further, these memory cells were apparently class-specific, that is, they induced in bone marrow cells antibody of only one immunoglobulin class, IgM. A secondary response to heterologous red cells in intact mice is usually characterized by a change in the relative amounts of IgG and IgM antibody produced (Adler, 1965), but it is apparent that this change is not an invariable feature of anamnestic responses.

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