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Antibody Production by 'Cloned' Cell Populations

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Summary. A method for the instrasplenic cloning of lymph node cells, based on the injection into X-irradiated recipients of cells from phytohaemagglutinin treated donors was developed. Clones were tested for their capacity to form anti-Shigella antibodies, and to produce a graft versus host immune response. It was found that each of the randomly chosen clones produced antibodies to Shigella. When immunized 'cloned' cell populations were injected into secondary recipients, and the latter were again immunized with Shigella, an increased response was obtained. Clones produced by bone marrow cells in spleens of X-irradiated animals, similarly tested for the immunological competence did not produce antibodies to Shigella. Cells of lymphoid clones of parental strain origin produced a graft versus host response in newborn F_1 hybrids. Bone marrow derived clones did not. Thus, each cloned cell population appears to be *a priori* pluripotential with respect to antibody formation. The immunological findings, analysed in relation to the population sizes of the tested clones, seem to indicate that each immune competent cell may, in fact, be pluripotential.

INTRODUCTION

The question whether each immune competent cell within one organism is genetically determined to form a single specific antibody, or whether it is *a priori* pluripotential, capable of forming antibodies to any given antigen, presents a crucial and as yet unsolved problem. The first alternative has been implied by the clonal selection theory of antibody (Burnet, 1959; Lederberg, 1959). The second, i.e. the state of pluripotentiality, may envisage either an instructive mechanism or a state in which all antibodies are determined by specific genes, but the entire spectrum of such genes is located within the genome of each competent cell (Szilard, 1960). The fact that following immunization with two different Salmonella antigens, each cell was found to produce antibodies to only one antigen at a time (Mäkelä and Nossal, 1961; Nossal and Mäkelä, 1962), does not imply that these cells were predetermined to produce only this antibody. It is conceivable that pluripotential cells, once activated by a particular antigen, may become unresponsive to other antigenic determinants. Indeed, some degree of pluripotentiality has been indicated in the results of antibody production to phage antigens by single cells (Attardi, Cohn, Horibata and Lennox, 1959; Attardi *et al.*, 1964a, b, c). A direct approach to test whether cells *a priori* can react to any given antigen has been attempted in the present study, based on a system for the *in vivo* production of clones of lymphoid cell populations, developed in our laboratory (Mekori, Chieco-Bianchi and Feldman, 1965). Clones of lymphoid cells were produced in spleens of X-irradiated mice by injecting lymph node cells from phytohaemagglutinin-treated animals. Randomly

selected clones were tested for their capacity to form antibodies to Shigella antigen, or to elicit a graft versus host response in newborn F_1 hybrid mice. The results appear to indicate that each cloned cell population is capable of forming an immune response to the antigens tested, providing evidence that each immune cell is, in fact, pluripotential.

MATERIALS AND METHODS

Animals

Twelve-week-old mice, of strains (C3H × C57Bl)F_1 and C57Bl were used in the present study.

X-Irradiation

Mice were exposed in Lucite containers to a lethal dose of 850 r total body irradiation, using a General Electric Maximar III X-ray machine (250 kV, 15 mA), with 0·5 mm copper and 1 mm aluminium filters, at a target distance of 50 cm (dose rate, 65 r/min).

Production of lymphoid clones

The method for production of intrasplenic colonies of lymphoid origin has been described elsewhere (Mekori *et al.*, 1965). Prospective donors of lymphoid cells were injected intravenously with 0·3 ml phytohaemagglutinin M (PHA, Difco). Three days later, the animals were killed and the inguinal, brachial and mesenteric lymph nodes were excised. The lymph nodes were disrupted with scissors, and the cells were suspended mechanically, by pressing through a stainless steel mesh (porosity 0·6 × 0·6 mm), and then passing through a hypodermic syringe containing a metal mesh. After being suspended in Tyrode's solution, washed twice and centrifuged at 3000 rev/min for 10 minutes, they were injected intravenously into X-irradiated animals at doses of 4 or 5×10^7 cells (see 'Experimental' section). The intrasplenic colonies formed were excised from the spleens for further treatment on Day 12 following cell inoculation.

It was previously demonstrated (Mekori *et al.*, 1965) that there was a linear relationship between the dose of inoculated lymphoid cells and the number of colonies obtained in each experiment, thus indicating that a colony can be initiated by a single cell. This was further substantiated by testing the agreement between the incidence of spleen colonies, in different experiments, and the Poisson distribution. It was found (Table 1) that the distribution of colonies per spleen fits the hypothesis of a Poisson distribution since the variance is not much different from the mean.

Production of clones of bone marrow origin

This was carried out by intravenous injection into lethally irradiated mice of bone marrow cells from tibias and femurs of 3-month-old donors (Till and McCulloch, 1961).

Antibody production and assay

Shigella antigen was injected intraperitoneally in doses of 0·2 ml of 0·1 per cent v/v alcohol-killed *Shigella dysenteriae* (courtesy of Dr T. N. Harris). X-irradiated animals inoculated with lymph node cells were immunized 7–9 days following cell injection. On Day 12, the animals were killed and their spleen colonies removed; the cells of each individual nodule ($1–2 \times 10^6$ cells) were suspended in saline, and injected intravenously into a second X-irradiated recipient a few hours following exposure.

TABLE 1

DISTRIBUTION OF SPLEEN COLONIES IN INDEPENDENT EXPERIMENTS

Exp. No.	No. of cells injected	Colonies per spleen	Mean	Variance	Mean/ Variance
1	4×10^6	0, 0, 0, 0, 1, 0, 0, 0, 0	0·10	0·10	1·00
2	20×10^6	1, 0, 0, 0, 1, 2, 1, 0, 1, 0, 1, 0	0·58	0·44	1·31
3	40×10^6	0, 0, 0, 0, 0, 1, 1, 2, 0, 0, 0, 0, 2, 3, 0, 0, 0, 1, 0, 0, 1, 2, 1, 2	0·67	0·84	0·79
4	40×10^6	0, 0, 1, 2, 0, 2, 1, 0, 3, 0, 1, 2, 0, 2, 3, 0, 0, 0, 1, 0, 0, 1, 0, 0, 2, 0, 0, 0	0·75	1·00	0·75
5	50×10^6	1, 3, 3, 4, 1, 4, 3, 4, 1, 2, 2, 0, 3	2·38	1·75	1·36
6	50×10^6	4, 4, 2, 3, 2, 5, 4, 2, 2, 5, 1, 2, 6, 2, 3, 8, 4, 1, 2, 6, 5, 0, 4, 3, 4, 8	3·51	3·97	0·88

Differences in the clone forming units/10^6 cells are obtained when different pooled cells are used in the different experiments. In a single experiment, using cells from one pool, there is a linear relationship between cell dose and colony numbers (Mekori *et al.*, 1965).

The second recipient received, in most experiments, a further immunization with Shigella antigen, 5 days after cell inoculation. The secondary recipients, whether immunized or not, were bled on Day 11 following injection of the cloned cell populations. The sera were tested for agglutinin content by making serial two-fold dilutions, and incubating each with 0·05 per cent antigen first for 1 hour at 37° and then for 48 hours at 4°. They were then examined for macroscopic agglutination.

Spleen assays of the 'Simonsen effect'

Newborn F_1 hybrid mice were injected intraperitoneally with 0·1 ml of a suspension of cells from clones of lymph node or bone marrow origin. The recipients were killed after 10–12 days, and the spleen indices were determined according to Simonsen, Engelbreth-Holm, Jensen and Poulsen (1958).

EXPERIMENTAL

Experiment 1

Experiment 1 was designed to test whether cloned cell populations of lymph node origin will produce antibodies to Shigella antigen. Thirty-eight ♀C57Bl mice (donor group a), were exposed to 850 r, and each was then injected with 4×10^7 cells from lymph nodes of animals pretreated with PHA. Ten ♀C57Bl mice (donor group b), were similarly irradiated but did not receive any cell inoculation. Seven days after irradiation, the animals of both groups and ten normal syngeneic animals (donor group c) were immunized with Shigella antigen. Five days later (i.e. on Day 12 following X-irradiation of donor groups a and b), the animals were killed, and the spleens excised. In the four survivors of donor group a, the numbers of macroscopic nodules per spleen were two, three, one and two. No nodules were found in the X-irradiated animals which had not been injected with lymph node cells. Normal ♀C57Bl mice were now irradiated with 850 r, and divided into five groups.

Group 1: Eight animals were each injected intravenously with a suspension of cells from a single nodulus of donor group a.

Group 2: Seven animals were injected each with 8×10^6 spleen cells of individual animals of donor group b. (This was to check whether immune reactivity, if found in group 1, could have been due to transfer of competent cells from the spleen of the donor.)

Group 3: (twenty-five animals) and Group 4 (twenty-five animals) were inoculated with 5×10^5 and 1×10^6 bone marrow cells respectively, from individual animals of donor group c.

Group 5: Seven animals served as irradiated controls.

Five days after X-irradiation, the animals of all five groups were immunized with Shigella antigen and 6 days later their sera were tested for agglutinins. The results (Table 2) indicate that the only group which produced antibody to Shigella antigen

TABLE 2

PRODUCTION OF AGGLUTININS TO SHIGELLA ANTIGEN BY CLONED CELL POPULATIONS—EXPERIMENT 1

Group No.	Treatment:* X-irradiated mice inoculated with	No. of reacting animals/No. of survivors	Per cent reacting	Titre $\log_2$ produce by animal No.						Mean titre $\log_2$
				1	2	3	4	5	6	
1	Immunized lymphoid clones (from donor group a)	5/6	83	10	9	0	7	5	7	6·33
2	8×10^6 spleen cells (from donor group b)†	0/6	0							0
3	5×10^5 bone marrow cells‡	0/14	0							0
4	10^6 bone marrow cells‡	0/17	0							0

* All animals were injected with Shigella antigen 5 days following cell inoculation.
† From immunized X-irradiated mice.
‡ From normal immunized mice.

was the one in which the animals were inoculated with the cloned cells of lymph node origin. Five out of six animals, each of which had received cells of different individual clones, showed agglutinating antibodies, with a mean log titre of 6·33. The antibodies measured in this group could not be attributed to the presence of background spleen cells which may have been transferred with the nodules, since Group 2, inoculated with much higher doses of spleen cells (of donor group b, immunized similarly to donor group a), did not show any detectable antibody. Neither could the antibody production be attributed to immunologically competent cells of the secondary recipients, since Groups 3 and 4, which were effectively protected with bone marrow cells from immunized donors and were then themselves immunized, did not produce antibody. It is therefore concluded that five out of six clones tested in this experiment produced antibody to Shigella antigen.

Experiment 2

Experiment 2 was aimed at comparing the immune response of cloned cells which were in contact with antigen only once, in the primary recipient, to that of clones which were in contact with antigen both in the primary and secondary recipients. Having found that cell populations of clones derived from lymph nodes are capable of producing antibody to Shigella antigen, whereas bone marrow cells, even from immunized animals, are not, attempts were made to improve the survival of the secondary X-irradiated recipients by injecting bone marrow cells in addition to populations of cloned lymphoid

cells. To eliminate the possibility that the administered bone marrow cells were participating in the immune response, tests were made in which control X-irradiated animals were injected with bone marrow from immunized donors, then further challenged with Shigella antigen. The following procedures were thus carried out: Forty-four X-irradiated ♀(C3H × C57Bl)F_1 animals were injected each with 4×10^7 lymph node cells from PHA treated donors (donor group a); twenty-three mice served as irradiated controls (donor group b). On Day 6, the two groups, and in addition ten normal animals (donor group c), were immunized with Shigella antigen. On Day 11, sixty-eight secondary recipients were X-irradiated, and divided into five groups.

Group 1: Eighteen animals were each injected with a cell population from an individual lymphoid clone of donor group a, plus 1×10^6 syngeneic bone marrow cells from normal donors.

Group 2: Nine animals were each injected with 8×10^6 spleen cells from donor group b and 1×10^6 normal bone marrow cells.

Group 3: Twenty animals were each injected with 1×10^6 normal bone marrow cells.

Group 4: Eleven animals were each injected with 2×10^6 bone marrow cells from donor group c.

Group 5: Ten of the irradiated animals served as controls.

On Day 16, nine animals of Group 1 (designated as 1a), were injected with Shigella antigen. On Day 21, all animals were bled and the individual sera were tested for agglutinin to Shigella. The results (Table 3) show that: (a) each of the lymphoid clones tested produced antibodies to Shigella, whereas none of the controls showed any detectable antibody formation; and (b) cloned cell populations which were challenged twice with the antigen (Group 1a) rather than once (1b), showed a higher titre of agglutinating antibodies (7·7 as compared to 4·7), i.e. a secondary immune response.

TABLE 3

PRODUCTION OF AGGLUTININS TO SHIGELLA ANTIGEN BY CLONED CELL POPULATIONS—EXPERIMENT 2

Group No.	Treatment: X-irradiated mice inoculated with	No. of reacting/No. of survivors	Per cent reacting	Titre, $\log_2$ produced by animal No.												Mean titre $\log_2$
				1	2	3	4	5	6	7	8	9	10	11	12	
1	Immunized lymphoid clones and 10^6 bone marrow cells	8/8	100	5	5	6	7	5	5	3	2					4·75
1a	Immunized lymphoid clones, 10^6 bone marrow cells then 2nd immunization	9/9	100	10	9	6	7	5	8	4	10	10				7·66
2	8×10^6 spleen cells*, and bone marrow cells, then Shigella antigen	0/8	0													0
3	10^6 bone marrow cells†, then Shigella antigen	0/18	0													0
4	2×10^6 bone marrow cells†, then Shigella antigen	0/10	0													0
5	Shigella antigen	0/3	0													0
6	Normal control, Shigella antigen	12/12	100	10	10	9	10	4	5	6	9	10	10	7	7	8·08

* From immunized, X-irradiated donors.
† From immunized normal donors.

Experiment 3

In the previous experiments, the primary antigenic stimulation to the cloned cells was applied 5 days prior to cell transfer, i.e. when the clonal populations were already established, but replication of the cloned cells would be expected to continue for a few more days. In the following experiment, the primary antigenic challenge was given to 8-day-old clones, i.e. to fully developed cloned populations, and the cells were transferred to secondary recipients 3 days later. The results (Table 4), again indicated that each of the clones thus tested formed agglutinins, whereas none of the controls showed antibody production. In this case also the cloned populations which were immunized twice manifested a higher titre than those immunized once only with the antigen.

TABLE 4

PRODUCTION OF AGGLUTININS TO SHIGELLA ANTIGEN BY CLONED CELL POPULATIONS—EXPERIMENT 3

Group No.	Treatment: X-irradiated mice inoculated with	No. of reacting/No. of survivors	Per cent reacting	Titre, $\log_2$ produced by animal No.										Mean titre $\log_2$
				1	2	3	4	5	6	7	8	9	10	
1	Immunized lymphoid clones and bone marrow cells	5/5	100	5	4	5	4	4						4·40
1a	Immunized lymphoid clones bone marrow cells, then 2nd immunization	9/9	100	5	5	8	6	6	6	6	5	6		5·88
2	8×10^6 spleen cells*, then Shigella antigen	0/7	0											0
3	10^6 bone marrow cells†, then Shigella antigen	0/10	0											0
4	2×10^6 bone marrow cells†, and Shigella antigen	0/8	0											0
5	Normal control, Shigella antigen	10	100	9	9	9	10	8	10	6	7	10	8	8·60

* From immunized, X-irradiated donors.
† From immunized normal donors.

Experiment 4

The previous experiments demonstrated that each of the cloned cell populations originating from lymph nodes were capable of producing antibodies to Shigella. The following series of tests were aimed at comparing the immunological competence of lymphoid clones with that of intrasplenic clones produced by bone marrow cells. Seventy ♀(C3H × C57Bl)F_1 hybrid mice were irradiated and injected, one group (donor group a, twenty-nine animals) with 4×10^7 lymph node cells and the second (donor group b, thirty animals) with 10^5 bone marrow cells (to produce clones of bone marrow origin). The third group (donor group c) served as irradiated controls. On Day 8, all groups were immunized with Shigella antigen, and 3 days later the animals were killed, and the spleens excised. A further seventy ♀(C3H × C57Bl)F_1 animals were now irradiated, and divided into five groups as follows: (1) fourteen animals were each injected with the cell population of a single lymphoid clone from donor group a; (2) twenty-six animals were each injected with the cell population of a single bone marrow-derived clone, from donor group b; (3) ten animals were injected with spleen cells of donor group c; (4) ten animals were injected with spleen cells from animals of donor group a, which did not show the formation of lymphoid nodules. This group was chosen

in order to compare the competence of the clones with that of the host spleen cells which could have changed their immune competence due to the injection of lymphoid cells; and (5) ten animals were X-irradiated without cell inoculation. Two days later, seven animals of Group 1 (1a), sixteen of Group 2 (2a), and Group 5 were immunized with Shigella antigen, and tested for antibody 6 days following immunization. The results (Table 5) show that only clones derived from lymph node cells are capable of forming agglutinins to Shigella. No antibody could be detected in animals inoculated with bone marrow-derived clones. This is in accordance with our earlier demonstration of the immunological incompetence of cloned cell populations produced by bone marrow or spleen cell inoculation into X-irradiated animals (Mekori and Feldman, 1965).

TABLE 5

PRODUCTION OF AGGLUTININS TO SHIGELLA ANTIGEN BY CLONED CELL POPULATIONS—EXPERIMENT 4

Group No.	Treatment: X-irradiated mice inoculated with	No. of reacting/No. of survivors	Per cent reacting	Titre, $\log_2$ produced by animal No.							Mean titre $\log_2$
				1	2	3	4	5	6	7	
1	Immunized lymphoid clones	7/7	100	5	4	4	6	5	4	5	4·71
1a	Immunized lymphoid clones, then 2nd immunization	5/5	100	10	8	8	4	8			7·60
2	Immunized bone marrow clones	0/10	0								0
2a	Immunized bone marrow clones, then 2nd immunization	0/12	0								0
3	8×10^6 spleen cells*, then Shigella antigen	0/8	0								0
4	8×10^6 spleen cells from donor group a, then Shigella antigen	0/10	0								0
5	Shigella antigen	0/4	0								0

* From immunized X-irradiated donors.

Experiment 5

The previous experiments demonstrated the immunological competence of cloned cell populations as manifested by the production of agglutinins to Shigella antigen. Furthermore, the clones were stimulated by the antigen 3–5 days prior to their removal from the spleen. The following experiment was aimed at testing the immunological reactivity of cloned cells in a completely different system, namely a primary graft versus host response. The clones were dissected out, and the immunological reactivity of individual clones of parental strain origin was tested for production of splenomegaly in newborn F_1 hybrids. Forty-eight ♀C57Bl animals were irradiated and each injected with 5×10^7 lymph node cells from animals pretreated with PHA. A further five irradiated control mice were not injected. On Day 11, the surviving fourteen test animals showed the following distribution of spleen nodules: 1, 4, 6, 8, 7, 5, 7, 9, 8, 8, 11, 10, 9, 9. The nodules were dissected out, and cell suspensions prepared. Two litters each consisting of seven newborn $(C3H\times C57Bl)F_1$ hybrid mice were used for testing manifestations of graft versus host response following the injection of cell populations from clones of parental strain origin. In each litter, four animals were each injected with 5×10^6 cells, pooled from two to three individual nodules. (Populations from single clones appeared

to be too small to elicit a measurable splenomegaly.) The other three newborn animals served as untreated controls. Eleven days following cell inoculation, the surviving animals (four experimental and four control) were killed, and the spleen indices determined. The results (Table 6) indicate that cells from cloned populations did elicit splenomegaly in F_1 hybrids, which seems to imply a graft versus host immune response.

TABLE 6

INDICES OF GRAFT REACTION C57Bl→(C3H × C57Bl)F_1 PRODUCED BY CLONED LYMPH NODE CELLS—EXPERIMENT 5

	Body weight (g)		Spleen weight (g/100 g)	
Normal control	5·9		0·510	
	5·4		0·370	
	6·0		0·320	
	5·8		0·300	
Mean	5·7		0·375	
	Body weight		Spleen weight	
	(g)	Index	(g/100 g)	Index
Experiment	6·4	1·12	0·690	1·84
	6·1	1·07	1·050	2·69
	5·8	1·01	0·640	1·70
	5·6	0·98	0·920	2·45
Mean				2·17

TABLE 7

INDICES OF GRAFT REACTION C57Bl→(C3H × C57Bl)F_1 PRODUCED BY CLONED LYMPH NODE AND BONE MARROW CELLS—EXPERIMENT 6

	Body weight (g)		Spleen weight (g/100 g)	
Normal control	4·40		0·380	
	4·50		0·270	
	4·45		0·320	
	5·20		0·390	
	5·80		0·470	
Mean	4·87		0·366	
Experiment	Body weight		Spleen weight	
	(g)	Index	(g/100 g)	Index
Bone marrow clones	4·80	0·985	0·350	0·956
	4·90	1·006	0·420	1·114
	5·10	1·047	0·370	1·010
	4·60	0·944	0·340	0·928
Mean				1·005
Lymphoid clones	5·00	1·026	0·960	2·622
	4·80	0·985	1·050	2·868
	5·10	1·047	0·980	2·677
	4·90	1·006	1·050	2·868
	5·20	1·067	1·100	3·005
Mean				2·800

Experiment 6

The capacities of parental strain clones of lymph node and of bone marrow origin to elicit splenomegaly in newborn (C57Bl × C3H)F_1 hybrid mice were compared in this experiment. Irradiated C57Bl animals were injected as follows: fifty with 5×10^7 lymph node cells from donors pretreated with PHA, twenty with 3×10^5 bone marrow cells, and ten served as unirradiated controls. On Day 11, the spleen colonies were dissected out, and cell suspensions from pooled individual nodules were injected into newborn animals from two litters. Five animals of litter 1 were each injected with 5×10^6 lymph node-originated clones, and three served as non-injected controls. Four animals of litter 2 were injected with 5×10^6 cells of bone marrow-originated clones, and two served as non-injected controls. On Day 11 following cell inoculation, the animals were killed and the spleen indices determined. The results (Table 7) show that cell populations of clones derived from lymph nodes elicited splenomegaly in newborn F_1 hybrids, whereas those derived from bone marrow did not. This would seem to confirm that cloned populations derived from lymph node cells are capable of manifesting a graft versus host immune response.

DISCUSSION

Recent studies of Haber (1964) have provided a clear demonstration that the tertiary structure and specificity of the antibody molecule is determined by the sequence of amino acids in the γ-globulin. Thus, the direct template theory of antibody formation, as suggested by Breinl and Haurowitz (1930), modified by Pauling (1940), and further formulated by Karush (1958) seems to have lost any factual basis. Yet, an instructional concept of antibody formation can still be conceived on the assumption of non-specific genes for immune-globulins in antibody-forming cells. The antibody specificity would then be conferred either by a mutation-like effect of the antigen (Schweet and Owen, 1957), or as seems more probable, would appear at a higher level of the protein synthesizing system, i.e. at the transcription or translation (Haurowitz, 1965) stages following the direct interactions of the antigen with, say, messenger RNA. According to such instructional concepts, each immunologically competent cell will *a priori* be pluripotential with regard to antibody specificity. On the other hand, following the prevailing dogma relating to genetic control of protein synthesis, it might seem probable that a specific gene exists for each specific antibody. Within such a conceptual framework, there are two alternative possibilities. Each immunologically competent cell may contain the entire spectrum of genes for all antibody specificities. This would—similarly to the 'instructional' theories—also imply that each antibody-forming cell is *a priori* pluripotential. Alternatively, as suggested by Burnet (1959) and Lederberg (1959), the cell population of an antibody-forming organ may be heterogeneous with respect to genes which determine immune-globulins, each cell carrying a gene for a different antibody. An antigen will trigger the replication of only those cells which carry the gene for its corresponding antibody, thus eliciting clones of cells capable of reacting to that specific antigen only. Although on the basis of some experimental results Burnet at one time revised the clonal selection theory (Burnet, 1961), in a more recent communication he has in fact returned to the original formulation of the clonal concept (Burnet, 1964), claiming for each immune competent cell a restricted potentiality in terms of antibody specificity.

The present study aimed at testing whether in fact immunologically competent cells are pluripotential, or whether each cell is predetermined for one specific antibody. This was carried out by cloning cells of lymph node origin prior to the antigenic stimulation, and then testing the immune response of individual clones to a given antigen. If each cell of a cloned population contains the genetic pattern of its original stem-cell (that is, if mutations did not alter the genetic structure of the population), the clonal selection concept would predict that of the randomly chosen clones practically none would react to a randomly chosen antigen, whereas the other two hypotheses would predict that each clone would be reactive to any antigen.

We have adapted the *in vivo* cloning system of Till and McCulloch (1961) for haemopoietic cells, to lymphoid cell populations. The method, which has been previously described (Mekori *et al.*, 1965), is based on the expected blastogenic effect of PHA on small lymphocytes (Mackinney, Stohlman and Brecher, 1962; Berman and Stulberg, 1962; Schrek and Rabinowitz, 1963). PHA-treated lymph node cells are injected into lethally X-irradiated mice. The colonies developed within the spleens of the recipient animals represent cloned populations derived from the injected cells. Trentin and Fahlberg (1963) have described the application of spleen cell-derived colonies for testing immunological reactivity of cloned cell populations, and claimed to have demonstrated immunological competence of such cell populations. In our experience, however, the cloning of spleen cells results always in erythroid, myeloid, or mixed (erythroid and myeloid) cell populations, which were found to be immunologically incompetent (Mekori and Feldman, 1965). In no instance did spleen cells produce colonies of lymphoid cell types unless the donors were pretreated with PHA. Since no rise in the mitotic rate of the cell populations of the lymph nodes due to phytohaemagglutinin was observed during 3 days following drug injection (unpublished observations), it appears probable that PHA transforms non-dividing lymphoid cells (possibly small lymphocytes) to blast cells, which, after settling in the spleens of the X-irradiated mice, are triggered to replicate by a mechanism analogous to that which applies in the case of stem cells of bone marrow origin.

Analysis of the cell composition of colonies produced by lymph node cells has not yet been completed. However, although the majority of cells represented within such noduli are of the lymphoid and plasma cells types, peroxidase stain has revealed the occurrence of cells of the erythroid line. If our tests using ^{59}Fe verify this finding, it will indicate that lymphocytes, following a 'blastogenic' transformation, may replicate and give rise to cells of both the lymphoid and erythroid lines. Although this possibility has been suggested by Yoffey and Courtice (1956) and by Cudkowicz, Bennett and Shearer (1964), no direct experimental method was available at the time to test it. Analysis of the differentiation of cloned cell populations has provided a tool for testing these suppositions; so far, the multipotentiality of the small lymphocyte in terms of differentiation of the lymphoid and erythroid cell lines is indicated.

The clones of lymph node-origin were tested for their immunological competence in two systems. Every clone was found to react to Shigella antigen, and every population of two to three pooled clones was found to give a positive Simonsen effect when tested on newborn F_1 hybrid mice. Although the Shigella antigen was injected 3 days prior to the excision of the clones, the fact that every clone was found to be reactive cannot be attributed to selection of pre-determined clones, since when the antigen was injected later, on the 8th day, all clones were already well established. Furthermore, the Simonsen

test excludes this possibility, since here the immunological competence was tested only after excision of the clones. Antibody formation by single cells of the lymphoid clones was also demonstrated in experiments (to be published, in collaboration with H. Gershon) in which individual cells forming sheep red blood cell haemolysin were measured by Jerne's plaque technique. It is therefore concluded that every clone is *a priori* pluripotential.

PHA has been demonstrated to be a weak antigen (Lycette and Pearmain, 1963; Norins and Marshall, 1964). However, even if PHA were acting as a strong immunogenic stimulus the conclusion regarding the immunological pluripotentiality of the clones produced by PHA would not be invalidated, but, if anything, substantiated. In this case a clone responding, apparently, to PHA would have been shown to react to other non-related antigens such as Shigella.

The question now arises whether *every cell* within a cloned population is pluripotential in terms of antibody production. Heterogeneity with regard to specific reactivity could have arisen within a cloned cell population, if the genes coding for immuno-globulins have a high mutation rate, as was suggested by Burnet (1959, 1964), and if mutational changes take place during cell replication within the intrasplenic colonies. However, even if one assumes a high mutation rate of 10^{-4} (Burnet, 1964) and a conservative estimate of 5×10^5 possible specific antibodies, one would not expect the tested clones all to respond to two randomly chosen antigens. A population of $1\text{–}2 \times 10^6$ cells would not accumulate a uniform distribution of such mutants. Therefore, the results so far obtained do not seem to be reconcilable with the notion that each immune competent cell is genetically predetermined to form one specific antibody, and our results are not in accord with the clonal selection theory of antibody formation. If, however, each immune competent cell is *a priori* pluripotential, one still has to choose between an instructional mechanism of antibody formation and the one based on the pre-existence, in every cell, of cistrons controlling a whole spectrum of antibody specificities.

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REFERENCES

ATTARDI, G., COHN, M., HORIBATA, K. and LENNOX, E. S. (1959). 'Symposium on the biology of cells modified by viruses or antigens. II. On the analysis of antibody synthesis at the cellular level.' *Bact. Rev.*, **23**, 213.

ATTARDI, G., COHN, H., HORIBATA, K. and LENNOX, E. S. (1964a). 'Antibody formation by rabbit lymph node cells. I. Single cell responses to several antigens.' *J. Immunol.*, **92**, 335.

ATTARDI, G., COHN, H., HORIBATA, K. and LENNOX, E. S. (1964b). 'Antibody formation by rabbit lymph node cells. II. Further observations on the behaviour of single antibody producing cells with respect to their synthetic capacity and morphology.' *J. Immunol.*, **92**, 346.

ATTARDI, G., COHN, H., HORIBATA, K. and LENNOX, E. S. (1964c). 'Antibody formation by rabbit lymph node cells. III. The controls for microdrop and micropipet experiments.' *J. Immunol.*, **92**, 356.

BERMAN, L. and STULBERG, C. S. (1962). 'Primary cultures of macrophages from normal human peripheral blood.' *Lab. Invest.*, **11**, 1322.

BREINI, F. and HAUROWITZ, F. (1930). 'Chemische Untersuchung des Prazipitates aus Hämoglobin und anti-hämoglobin Serum und Bemerkungen über die Natur der Antikörper.' *Z. physiol. Chem.*, **192**, 45.

BURNET, F. M. (1959). *The Clonal Selection Theory of Acquired Immunity*. Vanderbilt University Press, Nashville.

BURNET, F. M. (1961). 'Cellular aspects of immunology as manifested in the Simonsen reaction.' *Yale J. Biol. Med.*, **34**, 207.

BURNET, F. M. (1964). 'A Darwinian approach to immunity.' *Nature (Lond.)*, **203**, 451.

CUDKOWICZ, G., BENNETT, M. and SHEARER, G. M. (1964). 'Pluripotent stem cell function of the mouse marrow "lymphocyte".' *Science*, **144**, 866.

HABER, E. (1964). 'Recovery of antigenic specificity after denaturation and complete reduction of disulfides in a papain fragment of antibody.' *Proc. nat. Acad. Sci. (Wash.)*, **52**, 1099.

HAUROWITZ, F. (1965). 'Antibody formation and the coding problem.' *Nature (Lond.)*, **205**, 847.

KARUSH, F. (1958). 'Specificity of antibodies.' *Trans. N.Y. Acad. Sci.*, Ser. II, **20**, 581.

LEDERBERG, J. (1959). 'Genes and antibodies.' *Science*, **129**, 1649.

LYCETTE, R. R. and PEARMAIN, G. E. (1963). 'Further observations on antigen-induced mitosis.' *Lancet*, **ii**, 386.

MACKINNEY, A. A. JR., STOHLMAN, F. JR. and BRECHER, G. (1962). 'The kinetics of cell proliferation in cultures of human peripheral blood.' *Blood*, **19**, 349.

MÄKELÄ, O. and NOSSAL, G. J. V. (1961). 'Study of antibody-producing capacity of single cells by bacterial adherence and immobilization.' *J. Immunol.*, **87**, 457.

MEKORI, T. and FELDMAN, M. (1965). 'Protection of X-irradiated mice by "cloned" haemopoietic cells.' *Transplantation*, **3**, 98.

MEKORI, T., CHIECO-BIANCHI, G. and FELDMAN, M. (1965). 'Production of clones of lymphoid cell populations.' *Nature (Lond.)*, **206**, 367.

NORINS, L. C. and MARSHALL, W. H. (1964). 'Phytohaemagglutinin.' *Lancet*, **ii**, 648.

NOSSAL, G. J. V. and MAKELA, O. (1962). 'Elaboration of antibodies by single cells.' *Annu. Rev. Microbiol.*, **16**, 53.

PAULING, L. (1940). 'A theory of the structure and process of formation of antibodies.' *J. Amer. chem. Soc.*, **62**, 2643.

SCHREK, R. and RABINOWITZ, Y. (1963), 'Effects of phytohaemagglutinin on rat and normal leukemic human blood cells.' *Proc. Soc. exp. Biol. (N.Y.)*, **113**, 191.

SCHWEET, R. S. and OWEN, R. D. (1957). 'Concepts of protein synthesis in relation to antibody formation.' *J. cell. comp. Physiol.*, **50**, Suppl. 1, 199.

SIMONSEN, M., ENGELBRETH-HOLM, J., JENSEN, E. and POULSEN, H. (1958). 'A study of the graft-versus-host reaction in transplantation to embryos, F_1 hybrids, and irradiated animals.' *Ann. N.Y. Acad. Sci.*, **73**, 834.

SZILARD, L. (1960). 'The molecular basis of antibody formation.' *Proc. nat. Acad. Sci. (Wash.)*, **46**, 293.

TILL, J. E. and MCCULLOCH, E. A. (1961). 'A direct measurement of the radiation sensitivity of normal mouse bone marrow cells.' *Radiat. Res.*, **14**, 213.

TRENTIN, J. J. and FAHLBERG, W. J. (1963). 'An experimental model for studies of immunologic competence in irradiated mice repopulated with "clones" of spleen cells.' *Conceptual Advances in Immunology and Oncology*, p. 66. University of Texas, M.D. Anderson Hospital and Tumor Institute.

YOFFEY, J. M. and COURTICE, F. C. (1956). *Lymphatics, Lymph and Lymphoid Tissue*, 2nd edn, p. 422. Harvard University Press.