ANTIBODY PRODUCTION BY SINGLE CELLS: II. THE DIFFERENCE BETWEEN PRIMARY AND SECONDARY RESPONSE

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ONE of the unsolved problems in immunology is the nature of the difference between the primary and the secondary response. In a secondary response, far more antibody is formed ; this could be due to more cells producing antibody, to individual cells producing more antibody, or to a combination of these 2 factors. Burnet's (1957) clonal selection hypothesis of antibody formation envisages selective multiplication of clones of antibody-producing cells following antigenic stimulation. The secondary response could then be explained by the presence of a larger number of cells from the antibody-producing clone, resulting in a larger total output of antibody following further antigenic stimulation.

In previous communications (Nossal and Lederberg, 1958 ; Nossal, 1958) a technique was described for the detection of antibody production by single cells from hyperimmunized animals. This technique depended on the specific immobilization of *Salmonella* organisms by antiflagellar antibody. In the present experiments, the antibody output of isolated cells removed at the peak of either the primary or the secondary response was studied. It was shown that the chief difference between the primary and the secondary response was in the proportion of cells actively forming antibody.

MATERIALS AND METHODS

Animals.—Adult male Wistar albino rats were used. They were fed on a diet of cubes and tap water *ad libitum*.

Bacteria.—The monophasic organism *Salmonella adelaide*, flagellar antigen H_1^{fs}, was used. Bacteria were maintained at maximal motility by frequent passages through a semi-solid nutrient gelatin agar medium (Lederberg, 1956). Fresh 4 hr. broth cultures were used for motility-inhibition tests.

Antigen.—The antigen used was a preparation of isolated flagella from *Salm. adelaide*. The preparation of this antigen will be described in detail elsewhere. In summary, bacteria grown for 40 hr. on nutrient agar were suspended in saline, homogenized in a Waring blender for 3 min., and removed by centrifugation. The flagella present in the supernatant were deposited by high speed centrifugation in a Spinco ultracentrifuge. About 0·5 ml. of flagellar material was dissolved in 10 ml. of sterile physiological saline, and this solution was termed flagellar antigen.

Immunization of animals.—Rats were immunized by injecting 0·25 ml. of a 1 : 100 dilution of flagellar antigen in physiological saline into each hind foot-pad. Animals used for study of the primary response were killed 7 days after such injections. For the study of the secondary response, animals were similarly inoculated 4–6 weeks after the primary injections, and killed 6 days later.

Preparation of cell suspensions and micromanipulation.—The techniques used for the preparation of single cell suspensions from the popliteal lymph nodes draining the injection site

and for the isolation of these cells in microdroplets have been described in detail (Nossal, 1958). Several inprovements in technique have since been made, which are included in the following abbreviated description.

Both popliteal nodes from one animal were pooled, teased with needles in a Petri dish containing Earle's saline buffered to pH 7·0 with tris (hydroxy-methyl) amino methane (TRIS, Sigma & Co.), forced through a coarse sieve, and gently washed 3 or 4 times in Earle's saline by centrifugation. The final deposit of cells was suspended in sufficient Earle's saline supplemented with 30 per cent normal rat serum to make the concentration of cells approximately 10^6 per ml. In the studies of the primary response, this cell suspension was used for preparing microdroplets by free-hand manipulation. A coverslip was covered with medicinal paraffin oil, B.P. (H.C. Sleigh & Co.) and by the use of a fine Pasteur pipette, droplets containing 0 to 10 cells were deposited on the surface of the coverslip. The paraffin oil minimized evaporation. Such droplets contained about 10^{-5} to 10^{-6} ml. of fluid. The coverslip was then inverted over a de Fonbrune (1949) oil-chamber and incubated at 37° for 4 hr. In the secondary response studies, the final cell suspension was diluted to a concentration of approximately 2×10^4 cells per ml. with suspending medium and a large depot droplet, containing several hundred cells, was deposited on the coverslip. Numerous large droplets containing suspending medium only were also deposited. The coverslip was then inverted over the oil-chamber, and by micromanipulation under 100-fold magnification (dark-ground), single cells were drawn into the micropipette from the depot drop, washed in a clean droplet of suspending medium, and deposited on a known spot on the coverslip. Beside each droplet containing a single cell, a droplet of the cell's final washing fluid was placed for control purposes. About 40 such pairs of droplets could be prepared in about 1½ hr. The oil-chamber was then incubated at 37° for 4 hr.

The oil chamber was then placed on the microscope-stage, and a " cloud " of bacteria was inoculated into each droplet by micromanipulation. Owing to the large number of droplets present in each experiment, the exact number of bacteria in this cloud was not counted. but with increasing skill of the operator, rarely contained less than 7 or more than 10 bacteria. After 30 min. at room temperature, the droplets were surveyed for motility of the bacteria. Total loss of motility of all the bacteria was recorded as " inhibition ".

Titration of antibody content of microdroplets.—Droplets containing single cells showing inhibition were inoculated with a further cloud of bacteria, and observed at 5–10 min. intervals. As the number of active single cells in any experiment was small, greater accuracy was achieved here, and the number of bacteria introduced was actually 7–10. If at any time in the next 30 min. total immobilization of the bacteria had occurred, a third lot of 7–10 bacteria was introduced, and so on until the antibody content of the droplet had been exhausted.

The antibody content of the droplet was expressed as the number of lots of bacteria immobilized, and recorded as 1, 1½, 2, 2½, etc. For this purpose, the figure ½ meant partial immobilization of the final lot of bacteria added. The final lot of bacteria was considered to be partially immobilized if 1–3 bacteria remained motile 30 min. after introduction.

Control studies.—In the primary response studies the cell suspension before incubation, the suspending medium and numerous droplets prepared from the cell suspension but containing no cells were routinely tested for freedom from inhibitory activity. In the secondary response studies the suspending medium, single cell droplets before incubation, and the final washing fluid of each cell were similarly tested.

Serum titrations.—Serial two-fold dilutions of the sera from the rats studied were made in physiological saline containing 1 : 100 nutrient broth and 1 : 100 of a 4 hr. broth culture of *Salm. adelaide*. The concentration of organisms in this final suspension was 10^6 per ml. Samples of each serum dilution were transferred on to a glass slide under a layer of paraffin oil, and after 30 min. at room temperature, were surveyed for motility of the organisms. Ninety per cent immobilization was taken as the end-point and the reciprocal of the serum dilution giving 90 per cent immobilization was termed the titre of the serum.

RESULTS

The results of 12 experiments on the primary response are given in Table I and Fig. 1. Out of 662 single cells tested for antibody formation, 15 (2·3 per cent) were found to be active, and in 13 of these the titre of antibody was determined. It was 1 in 8 droplets, 1½ in 4 droplets and 2 in 1 droplet, giving a mean titre of 1·2.

TABLE I.—*Antibody Production in Droplets Containing Various Numbers of Primarily Stimulated Lymph Node Cells*

Experiment	Serum titre	Number of cells in each droplet						Titres‡ of active single-cell droplets
		1	2	3	4	5	6–10	
1	1,600	0*/10†	0/10	1/10	0/5	0/2	4/23	—
2	16,000	1/13	3/18	1/10	1/9	2/18	8/52	N.D.
3	16,000	1/52	2/26	1/18	1/16	2/14	10/56	1½
4	2,400	0/31	0/34	0/17	1/19	2/16	12/63	—
5	1,600	1/25	1/29	1/12	2/21	1/16	10/80	1
6	3,200	0/66	0/46	2/55	1/33	1/31	2/82	—
7	3,000	1/57	2/28	1/26	2/26	0/11	5/57	1
8	2,400	1/61	3/50	3/31	3/24	1/13	15/84	1
9	2,400	5/87	3/86	7/47	4/45	5/33	13/59	1, 1, 1, 1½, 2
10	1,200	0/91	2/65	0/59	0/37	1/19	3/79	—
11	1,600	3/52	3/32	2/19	4/19	2/6	5/24	1, 1½, N.D.
12	2,400	2/117	2/78	2/32	2/26	1/8	14/76	1, 1 (Mean = 1·2)
Total		15/662	21/498	21/336	21/280	18/187	101/735	
Percentage of active droplets		2·3	4·2	6·2	7·5	9·6	13·7	

* Numerator indicates number of droplets immobilizing *Salm. adelaide*.
† Denominator indicates number of droplets tested.
‡ Each unit of titre is equivalent to about 10 bacteria immobilized.
N.D. Not determined.

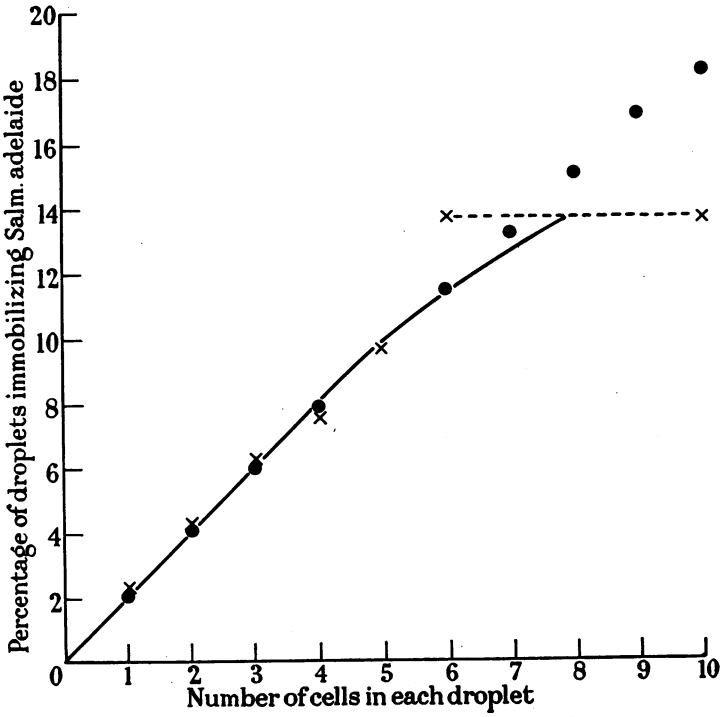

FIG. 1.—Antibody production in the primary response.

× indicates observed value.
×– – –× indicates observations on droplets containing 6 to 10 cells were pooled.
● indicated theoretical percentage if $n = 0{\cdot}02$ (see text).

Evidence of antibody formation was also sought in 2036 droplets containing from 2 to 10 cells. Fig. 1 shows the percentage of active droplets with various numbers of cells in each droplet. Observations on droplets containing 6–10 cells were pooled. The observed percentages are marked with crosses in Fig. 1. The significance of the dots in Fig. 1 is discussed below.

A further 6 experiments were performed on the secondary response. It was observed that the popliteal lymph nodes in these experiments were somewhat larger than in the previous experiments, and while no accurate estimation of this increase was attempted, the impression was gained that secondarily stimulated

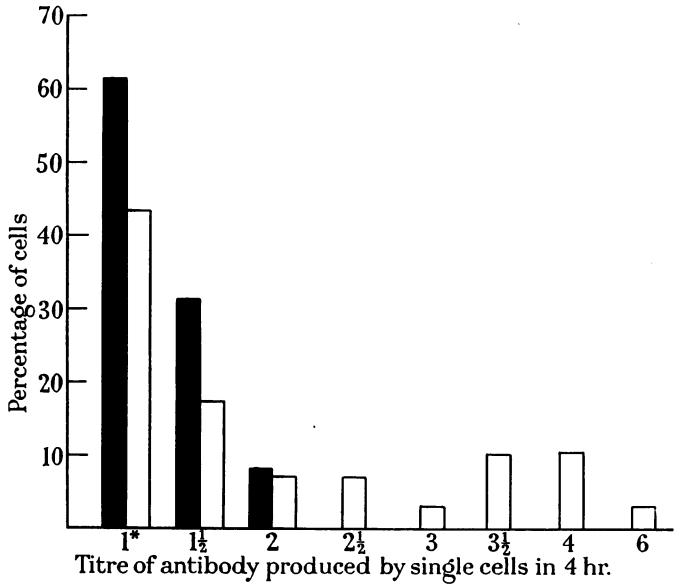

FIG. 2.—The titres of antibody formed *in vitro* by single cells removed during primary or secondary response.
* Each unit corresponds to about 10 bacteria immobilized.
■ indicates primary response.
□ indicates secondary response.

lymph nodes contained 1½–2 times as many cells as primarily stimulated nodes. Single-cell droplets in these experiments were prepared by micromanipulation in order that each cell might be given one individual wash in a large droplet of clean suspending medium. This ensured that the single cell droplets contained no antibody prior to incubation. The results obtained are given in Table II. Of 214 single cells studied, 30 (14 per cent) formed detectable antibody. The titres ranged from 1 to 6, with a mean of 2·0. Fig. 2 compares the titres of antibody formed *in vitro* by cells removed during either primary or secondary response. The difference in the mean antibody titre is statistically significant.

DISCUSSION

It is thought that the most important result of these experiments is the difference in the proportion of cells producing antibody in the primary and the secondary

response. It was observed that the percentage of active single cells was 6 times as high in the secondary response, and if the impression that the secondarily stimulated lymph nodes were at least $1\frac{1}{2}$ times as large as the primarily stimulated nodes was correct, this would mean that there were at least 9 times as many

TABLE II.—*Antibody Production in Droplets Containing Single Secondarily Stimulated Lymph Node Cells*

Experiment	Serum titre	Proportion of active cells	Titres‡ of active droplets
13	32,000	*9/46†	1, 1, 1, 1, 1, $1\frac{1}{2}$, $1\frac{1}{2}$, 2, $3\frac{1}{2}$
14	64,000	6/37	1, 1, 1, $1\frac{1}{2}$, $2\frac{1}{2}$, $3\frac{1}{2}$
15	16,000	1/25	$2\frac{1}{2}$
16	24,000	7/35	1, 1, 1, $1\frac{1}{2}$, 3, $3\frac{1}{2}$, 4
17	12,000	4/46	1, 2, 4, 6
18	4,000	3/25	1, $1\frac{1}{2}$, 4
			(Mean = 2·0)
Total		30/214	
Percentage of active cells		14	

* Numerator indicates number of droplets immobilizing *Salm. adelaide*.
† Denominator indicates number of droplets tested.
‡ Each unit of titre is equivalent to about 10 bacteria immobilized.

antibody-producing cells present in the secondarily stimulated nodes. This difference may be the major factor leading to the higher serum antibody titres obtained at the height of a secondary response. However, these conclusions are open to the possible criticism that a proportion of the cells in the primary response were making amounts of antibody too small to detect by the method used. Study of the results obtained in droplets containing 2–10 cells shows that this is not the case. On the hypothesis that the lymph node consisted of active and inactive cells, and that each active cell produced enough antibody to be detected by the test, the proportion of droplets containing 2 or more cells and showing inhibition would be given by the formula:

$$\text{Proportion} = 1 - (1 - n)^x$$

where n = the incidence of active cells, and x = the number of cells in each droplet. For small values of n and x this relationship is almost linear. In Fig. 1 the observed proportions (expressed as percentages) are plotted for values of x of 2–10. Also illustrated in this figure are the theoretical proportions expected from an incidence of active cells of 0·02 (2 per cent). It will be seen that the line of best fit drawn through the observed proportions coincides almost perfectly with the theoretical values. This seems to show that the method used was sufficiently sensitive to demonstrate antibody production by all the active single cells. A similar conclusion was reached when the hyperimmune response was studied (Nossal, 1958).

A factor contributing to the higher antibody titres observed in the secondary response may be the amount of antibody formed by the active cells in a given time. We observed that in 4 hr. active secondarily stimulated cells produced on the average 1·7 times as much antibody as active primarily stimulated cells. This greater production in a given time *in vitro* may or may not be a true indication of the rate of production *in vivo*.

The findings are in agreement with those of Leduc, Coons and Connolly (1955). In their study of the antibody content of cells by the fluorescent antibody technique, they found that in a secondary response, far more cells fluoresced than in a primary response and they fluoresced more brilliantly, implying greater antibody content.

The above workers (1955) also showed that the majority of cells in a lymph node draining the site of injection of diphtheria toxoid take up antigenic material within 6 hr. Of the current theories of antibody formation (Haurowitz, 1953 ; Pauling, 1940 ; Mudd, 1932 ; Burnet and Fenner, 1949 ; Jerne, 1955 ; Burnet, 1957) only one is directly relevant to the problem of why only a small proportion of these cells should be able to form antibody. This is Burnet's (1957) clonal selection hypothesis, which postulates that there are clones of antibody-producing cells, each clone being predestined to form one sort of antibody globulin molecule. Antigenic stimulation is followed by selective multiplication of representatives of the corresponding antibody-producing clone. It could be postulated that primary antigenic stimulation in our experiments was followed by limited multiplication of cells of the clone corresponding to *Salm. adelaide* flagellar protein, perhaps through 3 or 4 mitotic divisions ; secondary stimulation would then be followed by further multiplication, perhaps through 3 more mitotic divisions, to give a final proportion of 14 per cent of antibody-producing cells.

The author wishes to emphasize that Burnet's hypothesis is still highly speculative. While the above results are consistent with it, they by no means furnish proof. The exact theoretical significance of this work will only become clear when more is understood about the mechanism of antibody synthesis.

SUMMARY

Antibody production *in vitro* by isolated lymph node cells has been studied during the primary and the secondary response.

Rats were injected with isolated flagella prepared from cultures of *Salmonella adelaide* and the draining lymph nodes removed and processed to give single cell suspensions. Microdroplets containing single cells were prepared, and incubated for 4 hr. The amount of antibody formed by the cells was estimated by inoculating motile bacteria into the microdroplet and estimating the number immobilized.

Of 662 single cells tested in the primary response only 15 (2·3 per cent) formed detectable antibody. From the study of 2036 droplets containing 2–10 cells it was concluded that the 97·7 per cent of cells not forming detectable antibody were probably not making any antibody.

Of 214 cells tested in the secondary response, 30 (14 per cent) formed detectable antibody and the average amount formed by each cell was 1·7 times as much as in the primary response.

The theoretical implications of these findings are discussed in relation to Burnet's clonal selection hypothesis.

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