ANTIBODY PRODUCTION BY SINGLE CELLS

IV : Further Studies on Multiply Immunized Animals

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Earlier work from this laboratory (Nossal and Lederberg, 1958 ; Nossal, 1958) suggested that no cell will produce more than one type of antibody at one time. In more recent studies of antibody production by single cells (Nossal, 1959*a* ; 1959*b*) more satisfactory techniques for preparing single cell microdroplets were described. A better method for testing microdroplets for the presence of 2 or 3 unrelated antibodies is now reported. Using this method, we have tested 1399 lymph node cells from animals immunized in various ways with 2 or 3 unrelated antigens. The finding of phenotypic restriction of antibody-producing capacity of lymph node cells has been confirmed.

MATERIALS AND METHODS

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

Bacteria.—Four serologically unrelated monophasic strains of Salmonella were used. They were *Salmonella adelaide*, (H_1^{fg}), obtained from the Department of Public Health, University of Melbourne, through the courtesy of Mr. G. Cooper ; 2 transductional derivatives of *Salmonella paratyphi B*, Sw 685, (H_T^a) and Sw 997 (H_1^f) ; and 1 transductional derivative of *Salmonella typhi* Sw 940 (H_1^b). These 3 strains were kindly supplied by Professor Joshua Lederberg from his collection at Madison, Wisconsin. These bacterial strains and antigens derived from them will be referred to as AD, 685, 997 and 940 respectively. Bacteria were maintained at maximal motility by frequent passage through a semi-solid nutrient gelatin agar medium (Lederberg, 1956) and fresh 3–4 hr. broth cultures were used for motility inhibition tests.

Antigens.—Semi-purified flagella were prepared from each strain as previously described (Nossal, 1959*c*). About 0·5 ml. of flagellar material was dissolved in 10 ml. of sterile physiological saline and stored at −12°. These antigens were used at further dilutions of 1 : 10 to 1 : 500 in physiological saline.

Immunization of rats.—Various immunization schedules were employed. In all cases, rats received injections of 0·25 ml. of diluted antigen into each foot-pad. In some experiments, mixtures of 2 or 3 antigens were injected. In others, injection of one antigen was followed after some time by the injection of another antigen. Finally in some experiments, the antigen was incorporated into adjuvants, using Flozene 50 (H. C. Sleigh & Co.) as the mineral oil and Arlacel 80 (Purr-Pull Co.) as the emulsifying agent. Animals were killed 5–8 days after their last injection of antigen.

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 single cells in microdroplets closely followed the techniques previously described (Nossal, 1959*b*). The only change made was to select for study only those

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cells believed from their appearance at 100-fold magnification (dark-ground) to be plasma cells.

Estimation of antibody content of microdroplets.—The oil chamber carrying the single-cell microdroplets was incubated at 37° for 3½ hr. Then it was replaced on the microscope stage, and about 10 motile bacteria of one of the 2 or 3 immunizing strains were instilled into each microdroplet. If total loss of motility was observed at any time in the next 15–20 min. a further 10 bacteria of the same strain were instilled, and if they too were immobilized, a further 10 bacteria were instilled, and so on until the antibody content of the droplet had been exhausted or 5 lots of bacteria had been immobilized. The antibody titre of the droplet against this strain was then recorded as 0, 1, 1½, 2 etc. up to " 5 or greater ". For this purpose, the figure ½ meant partial immobilization of the final lot of bacteria added. The final lot was considered to be partially immobilized if 1–3 bacteria remained motile.

When the antibody titre of each droplet to the first immunizing strain had been determined, a small quantity of a 1 : 100 dilution of a specific antiserum to that strain was added to each droplet. This immobilized all the bacteria in all the droplets, providing a background against which the immobilization of further bacteria of the second immunizing strain could readily be tested. Then 10 bacteria of the second immunizing strain were added to each droplet and observed for immobilization. If immobilization occurred, the antibody titre of the droplet to that strain was determined as before. If 3 antigens had been used, the bacteria of the second strain were immobilized by a small quantity of specific antiserum as before, and the antibody titre against the third strain determined.

Staining of single cells.—In some experiments cells were stained with orcein and light green as previously described (Nossal, 1959*b*).

Serum antibody titrations.—The dilution of serum giving 90 per cent immobilization of a standard bacterial suspension was determined as previously described (Nossal, 1959*c*).

RESULTS

Assessment of antibody titration method

In early work on antibody production in doubly immunized animals (Nossal, 1958) only those cells capable of immobilizing the first strain were tested against the second strain. The amount of antibody formed against either strain was not determined. In the present studies, a new technique has been used to determine the antibody titre of all microdroplets against each of 2 or 3 strains of Salmonella. As outlined above, this involved instilling into the droplets a small quantity of specific antiserum to provide a background against which the immobilization of further, serologically distinct but morphologically indistinguishable, bacteria could be assessed. It was obviously important therefore to determine that the introduction of an excess of one antibody did not affect the titre of a second antibody present in the droplet.

Accordingly 2 immune sera were prepared against each of the 4 antigens by hyperimmunizing rats. Each serum was titrated against each strain of bacteria. These sera had titres of the order of 100,000 against the immunizing strain, but titres in the range <10–60 against the other strains. This indicated a very slight serological overlap between the strains. Next, microdroplets of the size usually used in single cell experiments were prepared containing the following dilutions of anti-AD serum in 30 per cent rat serum—Earle's saline : 1 : 100,000 ; 1 : 10,000 ; 1 : 5000 ; 1 : 2000 and 1 : 1000. Six droplets of each dilution were prepared, and to half of them, a small quantity of 1 : 200 anti-685 serum was added by micromanipulation. The titre of each droplet against AD was then determined. The results (Table I) showed that the presence of the anti-685 serum did not materially affect the anti-AD titre ; and that over the titre range 1–10, the anti-AD titre was approximately proportional to the dilution of immune serum.

TABLE I.—*Anti*-AD *Titres of Microdroplets of Standard Size Containing Diluted Anti*-685 *Serum*

Dilution of serum	Mean titre of droplets without anti-685	Mean titre of droplets with anti-685
1 : 100,000	<1	<1
1 : 10,000	1	1
1 : 5000	2·3	2
1 : 2000	4	4·7
1 : 1000	10·3	12.3
1 : 100	>20	>20

Furthermore, in the experiments to be reported each cell-containing droplet had beside it a control droplet containing only suspending medium and a small amount of washing fluid. The addition of antiserum to these droplets never affected the motility of bacteria of the second strain subsequently added to them.

Simultaneous double immunization with AD *and* 685.

Seventeen experiments were performed using mixtures of AD and 685 antigens. In these experiments the interval between primary and secondary immunization was 4–6 weeks and the animals were killed 5–8 days after secondary immunization. The relative doses of the 2 antigens varied widely (Table II).

TABLE II.—*Antibody Production by Single Cells from Rats Immunized with* AD *and* 685.

Expt.	Dilution AD antigen: 1ry. Immunization	Dilution AD antigen: 2ry. Immunization	Dilution 685 antigen: 1ry. Immunization	Dilution 685 antigen: 2ry. Immunization	Number of cells tested	Number of cells immobilizing AD	Number of cells immobilizing 685
1	1 : 100	1 : 100	1 : 100	1 : 100	30	13	2
2					40	19	1
3					22	5	1
4					33	10	0
5	1 : 100	1 : 100	1 : 100	1 : 10	39	5	2
6					32	12	1
7	1 : 100	1 : 100	1 : 25	1 : 100	18	0	3
8					27	5	1
9					37	5	1
10					32	9	1
11					21	2	0
12					36	2	1
13					29	2	0
14	1 : 50	1 : 500	1 : 50	1 : 50	24	3	4
15					30	1	2
16					36	1	2
17	1 : 100	1 : 100	1 : 10	1 : 10	33	9	0
Total					519	103	22

No cell produced antibody against both strains.

Of 519 cells examined, 125 produced antiflagellar antibody, 103 producing anti-AD and 22 anti-685, but no cell producing both antiflagellar antibodies. The titres produced by the single cells (Fig. 1) were well above threshold in most cases.

Simultaneous double immunization with other combinations

Six experiments were performed on rats immunized with mixtures of other combinations ; 2 with 685 and 997, 2 with AD and 997 and 2 with AD and 940. In other respects, these experiments were similar to the previous series. Of 210

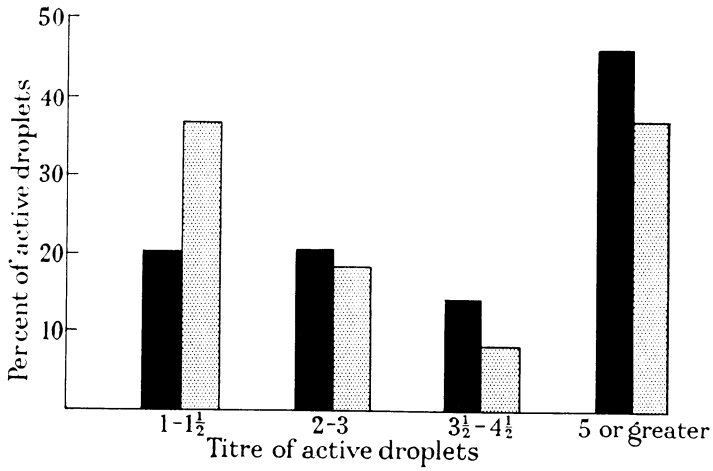

FIG. 1.—Titres of active single cell droplets from rats immunized with AD and 685.

Titres of droplets active against AD.

Titres of droplets active against 685.

cells examined, 53 produced antiflagellar antibody (Table III). No cell produced more than one antibody.

TABLE III.—*Antibody Production by Single Cells from Doubly Immunized Animals*

Expt.	Antigens	Number of cells tested	Number of cells active against	
			685	997
18 and 19	685 and 997	71	5	11
			AD	997
20 and 21	AD and 997	78	10	8
			AD	940
22 and 23	AD and 940	61	13	6
Total		210	53	

No cell produced antibody against both strains.

Simultaneous triple immunization

Ten experiments were performed in which the rats had been triply immunized with mixtures of AD, 685 and 997 antigens. In other respects, these experiments were similar to the previous series. All cells were tested against all three strains, and of 351 cells examined 104 produced detectable antibody (Table IV) ; 50 against

AD, 29 against 685 and 25 against 997. No cell produced antibody against 2 or all 3 strains.

The antibody titres produced by these cells are given in Fig. 2.

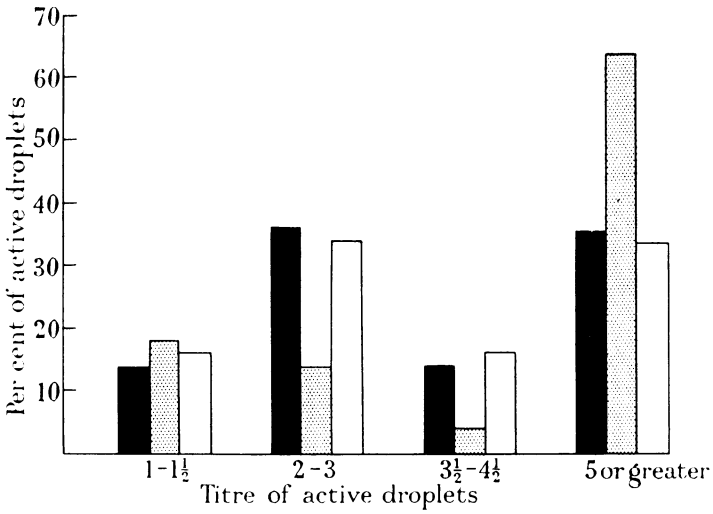

FIG. 2.—Titres of active single cells from rats immunized with AD, 685 and 997.

Titres of droplets active against AD.

Titres of droplets active against 685.

Titres of droplets active against 997.

TABLE IV.—*Antibody Production by Single Cells from Rats Immunized with Mixtures of* AD, 685 *and* 997 *Antigens*

Expt.	Number of cells tested	Number of cells immobilizing AD	Number of cells immobilizing 685	Number of cells immobilizing 997
24	36	7	5	3
25	33	7	4	3
26	43	10	5	3
27	29	4	5	3
28	31	1	0	0
29	36	2	1	5
30	30	1	3	2
31	35	4	1	3
32	36	9	1	2
33	42	5	4	1
Total	351	50	29	25

No cell produced antibody against more than 1 strain.

Sequential immunization

Five experiments were performed in which the rats had been immunized with the flagellar antigen incorporated in water-oil emulsion to give an adjuvant effect.

In one of these experiments (Expt. 34) injection of 685 in adjuvants was followed after 7 weeks by injection of AD in adjuvants, and the rat was killed 11 days later.

In 4 other experiments (Expt. 45–48), injection of 685 in adjuvants was followed after 7 weeks by injection of AD in adjuvants, and 2 weeks later by injection of 685 in saline. In all these experiments, the popliteal lymph nodes were greatly enlarged, being at least 10 times normal size. The results (Table V) show that out of 165 cells tested, 30 produced antibody ; 9 against AD and 21 against 685. No cell produced antibody against both strains.

TABLE V.—*Antibody Production by Single Cells from Rats Serially Immunized with* AD *and* 685 *in Adjuvants*

Expt.	Number of cells tested	Number of cells immobilizing AD	Number of cells immobilizing 685
34	27	4	0
35	35	2	2
36	32	1	5
37	36	1	6
38	35	1	8
Total	165	9	21

No cell produced antibody against both strains.

Five further experiments were performed on sequentially immunized rats. These received 2 pairs of injections of 940 in saline 4 weeks apart. They were injected with AD 2 days after the second injection and 4–6 weeks later, with a mixture of AD and 940. They were killed 5–6 days later. The results (Table VI) show that out of 154 cells tested, 35 produced antibody ; 27 against AD and 8 against 940. No cell produced antibody against both strains.

TABLE VI.—*Antibody Production by Single Cells from Rats Serially Immunized with* 940 *and* AD

Expt.	Number of cells tested	Number of cells immobilizing 940	Number of cells immobilizing AD
39	33	1	8
40	32	4	5
41	33	1	4
42	28	0	6
43	28	2	4
Total	154	8	27

No cell produced antibody against both strains.

Staining of cells

In about every third experiment, single cells were stained with orcein and light green. Of 337 identifiable cells, 291 (86 per cent) were plasma cells, and 17 (5 per cent) were plasmablasts. The remaining cells were medium and large lymphocytes (blasts) or polymorphs. Lymphocytes and polymorphs did not produce detectable antibody.

DISCUSSION

Since our previous report on antibody production by single cells from doubly immunized animals (Nossal, 1958), we have had much more experience of the various technical problems of single cell work, and have come to realize that the early experiments had the following serious limitations. Firstly, the amount of

antibody produced by the single cells was small. This was probably due to two reasons, namely the small size of the nutrient droplets and the early stage (3 days) after tertiary immunization at which the rats were killed. It is probable that the active cells at this stage were plasmablasts, which are not as active antibody-producers as plasma cells (Nossal, 1959*b*). Secondly, droplets may have contained small amounts of antibody prior to incubation because of inadequate washing of cells. Thirdly, comparatively few antibody-producing cells were tested. Finally, no attempt was made to test each droplet for antibody against each strain ; half the cells were tested against one strain and the other half against the other. Only droplets active against the first strain instilled were tested for antibody against the other strain.

In the present experiments, these difficulties have been overcome. Firstly, by the use of large microdroplets, and by concentrating study on more mature plasma cells taken 5–8 days after secondary immunization, it was found that most of the active cells produced amounts of antibody well in excess of the threshold (Figs. 1 and 2). Secondly, as each single cell was washed individually, the droplets before incubation were free of pre-formed antibody. Thirdly, by selecting for study only cells believed to be plasma cells we have been able to study far more antibody-producing cells. In all, 347 single-producers but no double-producers have been found. Finally, using the new antibody-titration method, the antibody content of each droplet against each bacterial strain could be assessed with reasonable accuracy (Table I, Figs. 2 and 3).

Although previous experience leads us to believe that 80–90 per cent of the cells selected in the present experiments were plasma cells (Nossal, 1959*b*), by no means all the cells produced antiflagellar antibody. Moreover, in most experiments, one antigen was more effective than the other (*e.g.* Table II). These results are the subject of further study, and will be discussed in the next paper of this series.

The present experiments confirm the observation that each antibody-producing cell can form only one antibody at one time. It is shown that out of 729 cells examined from animals immunized with mixtures of 2 antigens, 178 formed one or other antiflagellar antibody but none formed both (Tables II and III). Failure to find a double-producer does not mean that these do not occur. If the 729 cells are considered as derived from one population, failure to find a double-producer means that the incidence of double-producers in that population lies between 0 and 0·004 (E. S. Pearson and H. O. Hartley's Tables for 95 per cent confidence limits). We have studied a further 351 cells from animals immunized with 3 antigens, of which 104 formed one or other antibody. No double or triple-producers were found.

Other workers have approached this problem by the fluorescent antibody technique (Coons, 1958 ; White, 1958), which is capable of testing a large number of cells for the content of one or both of two antibodies. So far, no double-producers have been reported.

It has been suggested (Lederberg, 1959) that serial immunization of an animal first with one antigen and then with another could favour the multiplication of cells capable of forming both antibodies. To test this hypothesis, rats were immunized serially in 10 experiments. In the first 5 (Table V), rats received injections of the first antigen in adjuvants and 7 weeks later of the second antigen also in adjuvants. In the next 5 (Table VI), rats were given 2 injections of the first

antigen in saline 4 weeks apart, and then 2 days later, when extensive multiplication of antibody-forming cells would have been just beginning (Nossal, 1959*b*; Leduc, Coons and Connolly, 1955) the second antigen was injected. In 9 of these 10 experiments, the animals received a further injection before killing. No double-producers were found.

Following the injection of large amounts of 2 or 3 antigens into the foot-pad, it is likely that most cells in the draining popliteal lymph nodes encounter many molecules of both or all three antigens. There seem to be two main alternative explanations for the failure to find double or triple antibody producers. Firstly, it is possible that once a cell initiates antibody production corresponding to a particular antigenic determinant, the protein synthetic mechanism is pre-empted for that task, and incapable of producing a second antibody. Secondly, the secondary response might result from a clonal multiplication of specifically pre-adapted cells. Double immunization might lead to the proliferation of 2 distinct clones, one pre-adapted to form one antibody, the other to form the second. If this view were correct, it still remains to be determined whether the cells were pre-adapted *ab initio*, as in Burnet's (1957, 1959) clonal selection hypothesis, or whether they are the descendants of cells genetically modified by contact with antigen during the primary response.

SUMMARY

Further work on the phenotypes of antibody producing cells from animals immunized with 2 or 3 antigens is reported. Using a modification of previously described techniques for detecting antibody-production by single cells, cells from rats immunized with mixtures of 2 Salmonella flagellar antigens were tested for antibody production. Of these, 125 produced one or other antibody but none produced both. A further 351 cells from rats immunized with 3 antigens contained 104 single producers, but no double or triple producers. When rats were serially immunized with 2 antigens, similar phenotypic restriction of antibody-producing capacity was observed amongst 154 cells removed some weeks later.

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