SYMPOSIUM ON THE BIOLOGY OF CELLS MODIFIED BY VIRUSES OR ANTIGENS[1]

II. On the Analysis of Antibody Synthesis at the Cellular Level[2]

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The title of this symposium implies a similarity which is not obvious between the cellular responses to virus infection and to antigenic stimulation. In fact, no analogy between these two types of cellular response is apparent either from a consideration of the natures of the stimuli, a specific nucleotide sequence on the one hand and almost any foreign chemical configuration on the other, or from an examination of the products of the response, identical units in the case of the virus and complementary antibody units in the case of the antigen. Furthermore, so little is known about the mechanisms of the two responses at the chemical level that one would hesitate to compare them.

However, viruses, considered both as infective agents and as antigens, can induce in cells two types of response, the formation of identical units, virus particles, and of complementary units, antivirus antibodies. What are the properties of cells which condition their capacity to respond in one or the other or both ways? From this point of view, attention is focused on the ability of a cell to perform new syntheses, that is, to differentiate under the external stimulus of virus or antigen. It is in this way that the relationship between production of virus and antibody, implicit in the title of the symposium, can be justified; both phenomena provide a model for the study of cellular differentiation.

When virus and antibody production are considered as aspects of the same phenomenon, *i.e.*, cellular variation, it is not surprising that the methodology developed by virologists over many years should eventually become useful for the study of antibody production. In particular we are referring to that aspect of the methodology which is concerned with analysis of the nature and origin of the differences between single cells. Clearly, it is not necessary to justify studies on antibody synthesis in single cells to virologists. This would be like breaking down an open door.

Until recently antibody synthesis was studied by examining the changes in the serum or in the histological characteristics of the lymphatic tissue of an animal which had been exposed to a given antigen. The study of the serum during immunization revealed two extremely significant phenomena: the characteristic kinetics of antibody formation (reviewed in (1)) and the heterogeneity of antibody molecules (reviewed in (2, 3)). The kinetics of antibody synthesis in the animal is of an apparently autocatalytic type, subject to a large number of interpretations. It is essential to know whether this kinetics is the expression of an intrinsic property of the intracellular antibody-forming system, or whether, on the contrary, it merely reflects the time at which different antibody-forming cells start synthesizing and releasing antibody.

The same alternative between a populational and a cellular phenomenon holds for the interpretation of the heterogeneity of antibody molecules. Antibodies directed against a given antigenic determinant are markedly heterogeneous,

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not only with respect to the physicochemical properties of the γ-globulin with which antibody activity is associated, but also with regard to the specificity and combining power of the reacting sites. Again the problem arises whether the main source of variability resides in the properties of the antibody-forming system within each cell or whether, on the contrary, the heterogeneity of antibody has its origin at the level of the antibody-producing population, in which different cells synthesize slightly different molecules. It is pertinent to recall that enzymes, in contrast to antibodies, are relatively homogeneous in their combining properties (4). If antibody heterogeneity were due to differences between antibody-producing cells, then the situation would be analogous to having, for example, a mixed culture of a yeast and *Escherichia coli*, in which it would be found that a given enzymatic activity is associated with a heterogeneous protein fraction.

There exists today an enormous number of observations on the kinetics of antibody synthesis and on the heterogeneity of antibody molecules. Since antibody production by an animal may involve the activity of a heterogeneous population of cells, these findings cannot by systematized and interpreted until it is known at which level the analysis should be applied. Clearly the process must be traced back to its cellular origin, and the modifications in the individual cells following antigenic stimulation must be studied. Ideally, we should have a clone growing *in vitro*, induce it *de novo* to produce a given antibody, and then investigate this process in individual cells. Unfortunately, such a system is not yet available, although we should be optimistic and expect that we will not have to wait too long for it. Meanwhile, the closest alternative is to isolate antibody-producing cells from preimmunized animals and study antibody production in single surviving cells.

Any system designed for the study of antibody production by a single cell should satisfy the following requisites. (a) The assay should be sensitive enough not only to detect but also to give a quantitative estimate of the antibody produced by a single cell; (b) the background antibody activity should be low; and (c) the assay should be simple enough to allow the analysis of a large number of cells, in view of the possible heterogeneity of the antibody response by the individuals in a population of cells.

Experiments designed to investigate this problem have been reported by other investigators (5–8). We have developed a method for measuring the production of antibody by single cells isolated from rabbits hyperimmunized with bacteriophage (phage). The assay for antibody produced by the cells is based on the measurement of phage inactivation. This system has allowed us to obtain a considerable amount of information concerning the distribution of antibody-producing cells in a lymph node population from an immunized rabbit. In particular, our study has been directed toward determining (a) the proportion of lymph node cells producing a given antibody, (b) which morphological characteristics of a cell are associated with antibody synthesis, and (c) whether the synthetic capacity of a single cell is limited to only one antibody of a given specificity. The importance of the last question may not seem obvious at first, but we will have occasion to discuss it in detail later.

Let us begin by describing a typical experiment and then return later to a discussion of the controls which the results of this experiment will suggest. The rabbit used in this experiment had been hyperimmunized by injection, into the foot-pads, of the *E. coli* bacteriophages T2 and T5, which are serologically unrelated. A cell suspension was prepared from the popliteal lymph nodes by a procedure illustrated in figure 1. The lymph nodes were removed from the animal, cut into fairly large fragments, suspended in medium, and left under stirring for several hours at 0 C. During this time a large number of cells were released from the fragments into the medium. The cell suspension was filtered through a stainless steel wire gauze to separate the large fragments of lymph node, and then washed 5 times to dilute the free antibody present in the cell suspension. The cells were then isolated in two ways, either in microdrops of the order of 10^{-6} ml, or in micropipettes in a volume of about 10^{-3} ml.

Figure 2 shows the successive steps used to dispense the cells in microdrops and to recover the drops after incubation. An appropriate amount of the phages T2, T5, and T1 was added to the cell suspension, which was diluted to contain about 10^6 cells per ml. The phages T2 and T5, with which the rabbit had been immunized, served to test for antibody production in the microdrops by the neutralization of their

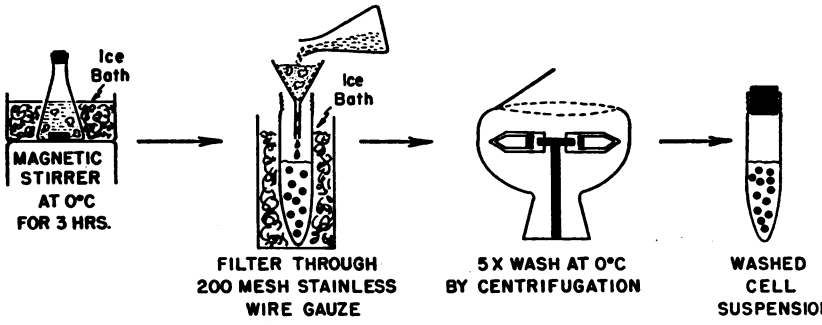

Figure 1. Schematic drawing illustrating the procedure used to prepare the cell suspension from the popliteal lymph nodes.

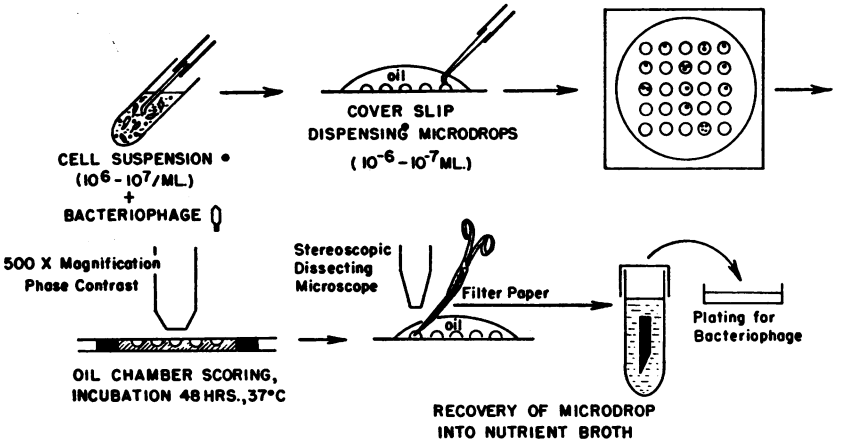

Figure 2. Schematic drawing representing the successive steps in an experiment utilizing the microdrop technique.

activity, whereas the phage T1, which does not cross react with antibody to T2 or T5, served as a microdrop volume indicator. An estimate of the volume of the microdrops was necessary for an evaluation of the amount of the phages T2 and T5 present at the beginning of incubation. Twenty-five microdrops were dispensed with a micropipette on each large cover slip covered with mineral oil. The cells distributed themselves in the microdrops so that some drops contained 0 cells, some 1 cell, and the others 2, 3, or more cells. The antibody produced by the cells was measured from the inactivation of the phages T2 and T5 in the individual drops; the drops with no cells were used to estimate the background inactivation. The number and type of the cells in the microdrops were recorded after examination by phase contrast microscopy. Following 48 hr incubation at 37 C the drops were recovered with thin strips of filter paper and diluted about 10^6-fold with nutrient broth. The broth was then plated with the indicator bacteria for phage assays. Each strain of phage was titrated on a different indicator bacterium.

Figure 3 shows the successive steps in an experiment utilizing micropipettes. Individual cells from a dilute suspension containing also the phages T2 and T5 were drawn up into carefully calibrated micropipettes. The micropipettes were broken at the point indicated in figure 3, then sealed with wax. After incubation, the contents of each micropipette were blown out into nutrient broth, which was then assayed for phage.

The microdrop and micropipette techniques do not differ in principle. In the former the advantage of great sensitivity associated with the high concentration of antibody attained in the small microdrop volume is reduced by the rather large background fluctuations due to volumetric uncertainties; in the micropipette technique, on the other hand, only low antibody concen-

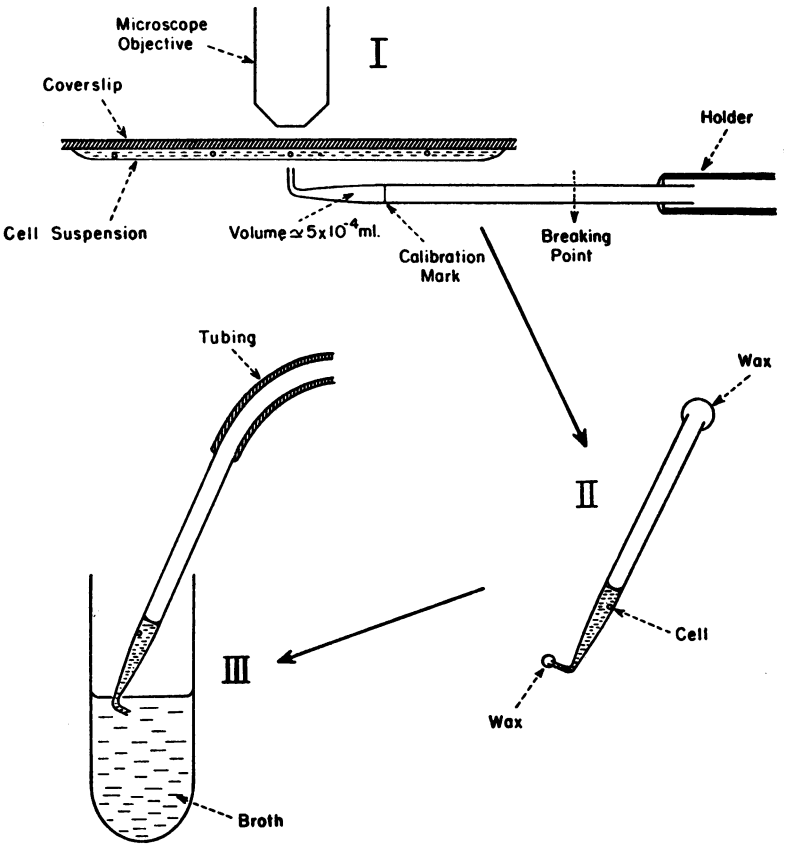

Figure 3. Schematic drawing representing the successive steps in an experiment utilizing the micropipette technique.

trations resulting from dilution in a comparatively large volume can be attained but they can be measured against very small background fluctuations.

The inactivation of phage by antibody is an exponential process described by the equation $P/P_0 = e^{-Kt/D}$ where P_0 is the initial phage titer, P is the phage titer after t minutes of contact with antibody, D is the dilution of the antiserum, and K is a constant that characterizes the rate of inactivation of a given phage by a given antiserum and gives a measure of the concentration of antibody.

The criterion for scoring a cell as an antibody producer in microdrop experiments is illustrated by the frequency histograms (figure 4), in which only the findings for T2 phage are presented. A logarithmic scale for the abscissae has been chosen to visualize the exponential relation between phage inactivation and antibody level. In the lower part of figure 4 is shown the frequency distribution of the T2 values, corrected for volume, in drops containing 0 cells. Correction for volume was made by dividing each T2 count by the corresponding T1 count, and multiplying the result by 100. This correction is not as good as we would like it to be, since there is still a marked spread of the individual values. A broken line has been drawn corresponding to $-3\ \sigma$ (σ = standard deviation) deviation from the average, that is, to a 0.001 probability level for a normal distribution.[7] This line has been

[7] The oversimplified criterion of $-3\ \sigma$ used here is somewhat misleading and will be discussed subsequently in a detailed paper. Here it suffices to say that the data from many of the experiments which we have performed do not fit a normal distribution and other criteria based on analysis of cumulative frequencies for 0-cell drops have been established which for this case give values for antibody-producing cells not very different from those described here.

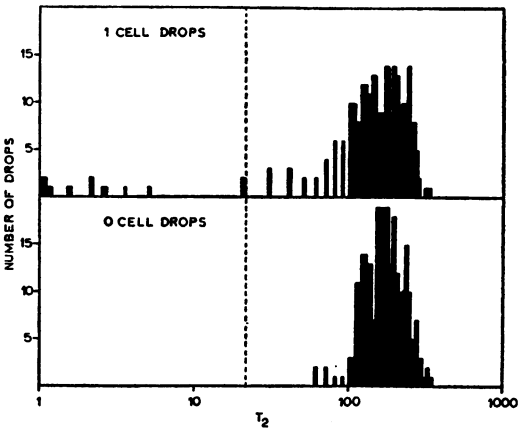

Figure 4. Frequency histograms of the T2 values, corrected for volume, of the drops containing 0 cells (*below*) and 1 cell (*above*). A broken line has been drawn corresponding to $-3\ \sigma$ deviation from the average of the T2 values for the 0 drops. Notice that whereas none of the T2 values of the 0 drops falls beyond this line, many T2 values of 1 cell drops outrange the $-3\ \sigma$ deviation from the control average.

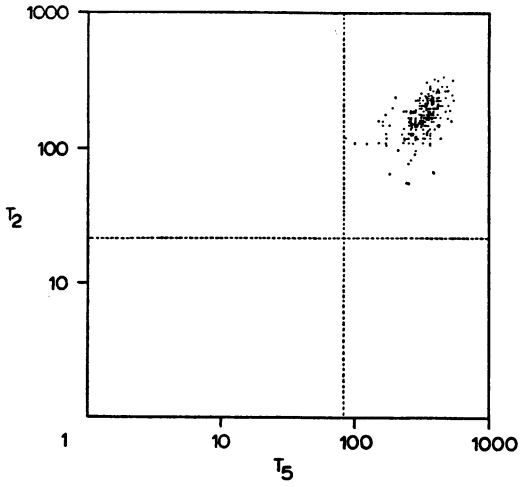

Figure 5. Scatter diagram of T2 *vs.* T5 values, corrected for volume, for the drops containing 0 cells. The lines corresponding to $-3\ \sigma$ deviation from the average for T2 and T5 have been drawn. Note that none of the values of the 0 drops falls beyond these lines.

chosen as the cutoff to define inactivation by antibody. Drops with values at the left of this line will be scored as containing antibody. None of the T2 values of the 0 drops falls beyond this line. In the upper part of the figure is found the frequency distribution of the T2 values, corrected for volume, of the drops containing 1 cell. In contrast to the 0 drops, many values fall beyond the line corresponding to $-3\ \sigma$ deviation from the control average and, therefore, have been interpreted to indicate inactivation by antibody. The background inactivation of phage corresponds to K of the order of 10^{-4}. In this experiment the values which fall beyond $-3\ \sigma$ correspond to inactivation constants $K > 10^{-3}$, which are at least 10 times greater than background. Results similar to those shown in this diagram were obtained for the phage T5 and identical criteria for defining antibody production have been used.

For the purpose of correlating the T2 values of the individual drops with the corresponding T5 values, it has proved convenient to represent the results in the form of a scatter diagram in which the individual T2 values, corrected for volume, are plotted against the corresponding T5 values, corrected for volume. Figure 5 shows such a type of a scatter diagram for the drops containing 0 cells. Here too, a logarithmic plot has been used. The lines corresponding to $-3\ \sigma$ deviation from the average for T2 and T5 are also drawn. One can see that the values of these 0 drops do not fall outside the $-3\ \sigma$ deviation from the average, either for T2 or for T5. This scatter diagram should be compared with the one of the drops containing 1 cell (figure 6). The values for many 1-cell drops fall beyond the line corresponding to $-3\ \sigma$ deviation from the control average for T2 or T5, or both phages. According to the criterion defined above, among 227 cells there are 10 anti-T2 cells, 7 anti-T5 cells, and 3 anti-T2,T5 cells. The points with a small arrow adjacent to the abscissae and ordinate axes represent drops in which there was complete disappearance of T2 or T5 particles. These points have been placed at the measurable limit of this experiment. The results we have just illustrated, including the data referring to the drops containing more than 1 cell, are summarized in table 1. The drops have been classified according to their cell content, and the number of anti-T2, anti-T5, and anti-T2,T5 drops, considered as three separate classes, have been tabulated. We will use these data as an example for the subsequent analyses.

Out of 925 lymph node cells examined, 95 cells, that is 10.3 per cent, produced detectable antibody in the microdrops (table 2). In other

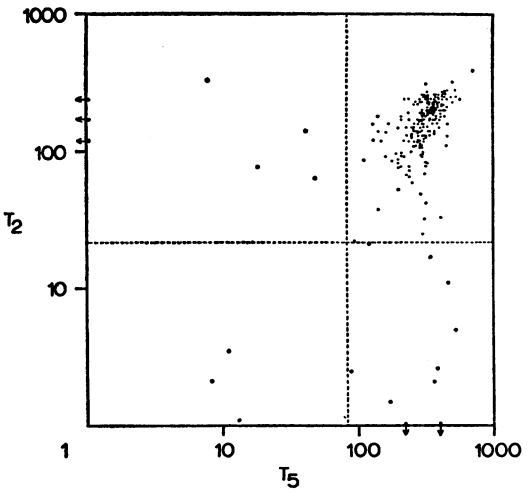

Figure 6. Scatter diagram of T2 *vs.* T5 values, corrected for volume, for the drops containing 1 cell. The lines corresponding to $-3\ \sigma$ deviation from the average for T2 and T5 in the 0 drops are also drawn. Compare figure 5, and notice that the values of many 1 cell drops fall outside the $-3\ \sigma$ deviation from the control average for T2 or T5 or both phages. The points with a small arrow adjacent to the abscissae and ordinate axes represent drops in which there was complete disappearance of T2 or T5 particles.

TABLE 1

Summarized results of the microdrop experiment described in the text

No. of Drops	Cells per Drop	Anti-T2	Anti-T5	Anti-T2,T5
220	0	0	0	0
227	1	10	7	3
165	2	19	12	8
76	3	8	5	10
35	4	8	2	1

TABLE 2

Distribution of antibody-forming cells in the lymph node population analyzed in the microdrop experiment of table 1

	Total Examined	Total Antibody Forming	Anti-T2	Anti-T5	Anti-T2,T5
No. of cells.....	925	95	47	27	21
Per cent of cells.	100	10.3	5.1	2.9	2.3

The number of anti-T2, anti-T5, and anti-T2,T5 cells was calculated from the number of inactivating drops and corrected for coincidence.

experiments the proportion of antibody-producing cells varied between 10 and 20 per cent.

If now we consider the different classes of antibody-producing cells, we notice that 5.1 per cent of lymph node cells produced anti-T2 antibody, and 2.9 per cent anti-T5 antibody. The most significant result, however, is that 21 cells out of 925, that is 2.3 per cent produced both anti-T2 and anti-T5 antibody. The value of 2.3 per cent for double producers is a minimum one, as is the figure of 10.3 per cent for the total proportion of antibody-producing cells. In fact, each of the strains of phage contains several antigenically different proteins and we have tested for antibody directed against only one of them, namely, the tail protein involved in the inactivation reaction. It is entirely conceivable, for example, that many anti-T2 cells and anti-T5 cells in this experiment, besides making antibody to tail protein, also produced antibody against the other phage proteins.

Now let us turn to another consideration. Since the *total* frequency of anti-T2 cells is 7.4 per cent and that of anti-T5 cells is 5.2 per cent, the probability of having a cell producing both anti-T2 and anti-T5 is 0.38 per cent (table 3), assuming that the induced syntheses of antibody to T2 and to T5 were two independent events liable to occur in any cell of the population (independence hypothesis). Since the frequency of anti-T2,T5 cells in this population is 2.3 per cent, much higher than would be expected under the independence hypothesis, one might be tempted to conclude that production of anti-T2 and anti-T5 is not independent but is, on the contrary, a linked event. However, a value of less than one for the ratio (F) of the calculated frequency of anti-T2,T5 cells to the found frequency, is compatible with several interpretations, of which only one will be mentioned here.

If it is assumed that the lymph node population is heterogeneous with respect to the potential antibody-forming capacity of the individual cells, *i.e.*, one fraction of the cells is capable of responding to antigen stimulation whereas the other is inert, then the independence hypothesis could still be valid. F, under this hypothesis, is equal to the proportion of the cell population

TABLE 3

Relationship between frequencies of anti-T2, anti-T5, and anti-T2,T5 cells in the lymph node population described in table 2

P_2 ≡ frequency of anti-T2 cells in total population = 0.074

P_5 ≡ frequency of anti-T5 cells in total population = 0.052

$P_{2,5}$ ≡ frequency of anti-T2,T5 cells in total population (found = 0.023; calculated under independence hypothesis = $P_2 \times P_5 = 0.074 \times 0.052 = 0.0038$).

Assuming heterogeneity of lymph node population (see text):

Let F ≡ proportion of potential antibody-formers in the population.

π_2 ≡ frequency of anti-T2 cells among potential antibody formers.

$$\pi_2 = \frac{P_2}{F}$$

π_5 ≡ frequency of anti-T5 cells among potential antibody formers.

$$\pi_5 = \frac{P_5}{F}$$

$\pi_{2,5}$ ≡ probability (or frequency) of anti-T2,T5 cells among potential antibody formers.

$$\pi_{2,5} \begin{cases} \text{based on observed } P_{2,5} = \dfrac{\text{observed } P_{2,5}}{F} \\ \text{expected under independence hypothesis} = \\ \quad \pi_2 \times \pi_5 = \dfrac{P_2}{F} \times \dfrac{P_5}{F} \end{cases}$$

$\therefore P_{2,5} = F \times P_2 \times P_5$, i.e.,

$$F = \frac{\text{Calculated } P_{2,5}}{\text{Observed } P_{2,5}} = \frac{0.0038}{0.023} = 0.17.$$

potentially able to make antibody as shown in table 3. As long as the observed $P_{2,5}$ is greater than the calculated $P_{2,5}$ then one can choose a value of $F < 1$ which would reconcile the two values. In the present case the data are compatible with the assumption that the population consist of 17 per cent potential antibody-producing cells and 83 per cent "inert" cells. Insufficient data are available to say whether this finding is of general significance.

Another interesting calculation can be made from these data. It should be recalled that antibody molecules have in general two combining sites per molecule (3, 9, 10). Moreover, the greatest part, if not all, of the antibody molecules in the serum have both sites specific for the same antigenic determinant, although the animal has been exposed to a large variety of different antigens; therefore, a mechanism must exist for excluding antibodies with double specificity, the so-called heteroligating antibody (11–14). The results of our experiments provide an indication as to the level at which this exclusion mechanism may operate. In fact (table 2), the largest proportion of antibody-producing cells, that is, 47 + 27 = 74 out of 95, or 78 per cent, produced antibody either against T2 or T5, and only 21 out of 95, that is, 22 per cent, produced both types of antibody. If in anti-T2,T5 cells, the anti-T2 and anti-T5 combining sites were formed in the same proportion as they are produced by the whole cell population, and if they were randomly distributed among the different antibody molecules within each cell, the number of single antibody molecules which could have different sites, i.e., an anti-T2 and an anti-T5 site, would be only 10 per cent of the total antibody molecules produced by the whole cell population. Were this the proportion of heteroligating antibodies present in the sera in previous experiments, they would have been difficult to detect by the methods applied; in fact it would not be necessary to change very much the figures obtained in the microdrop experiment analyzed above to go below the measurable limit in serological assays. Therefore, an exclusion mechanism for heteroligating antibodies does operate at the cellular level. It remains to be determined whether an additional exclusion mechanism also operates at the subcellular level.

An independent analysis was made of the distribution of antibody-producing cells in a lymph node population using the micropipette technique. The micropipette experiments gave results similar to those obtained in the microdrop experiments. Table 4 shows the results of 3 micropipette experiments made with rabbits immunized against both T2 and T5. The results of the microdrop experiment previously described are also tabulated. In the micropipette experiments, the cells selected under the microscope were mostly of the plasmacytic type. As we will discuss later, cells of this type show greater frequency of antibody producers than cells taken at random. This fact is clearly illustrated by the figures presented in tables 4 and 5. It should be noticed that a relatively high frequency of double antibody producers, that is 3 per cent, was found also in the micropipette experiments. The fact that comparable results were obtained with two independent methods gives greater validity to the findings.

TABLE 4

Comparison of micropipette and microdrop experiments with cells from rabbits immunized with bacteriophages T2 and T5

Class	Microdrops (Random Cells)	Micropipettes (Selected Cells) (3 Expts Summed)
	%	%
Anti-T2	5	10
Anti-T5	3	8
Anti-T2,T5	2	3
Total antibody-producing	10	21

It is pertinent to an analysis of the distribution of antibody-producing cells on a lymph node population to ask whether there is any correlation between morphological cell types and antibody-producing types. Since there might be some difference of opinion as to the nomenclature, the cell types to which we will refer are shown in figure 7, as phase-contrast photographs. The name stem, or blast cells, will indicate cells like *A* in figure 7. These are large cells with a large nucleus and a rather abundant cytoplasm, which is markedly basophilic on staining. We will refer to cells, such as *B* and *C*, as being of the plasmacytic series. Cell *B* can be considered as an immature plasmacyte, whereas cell *C* is a typical mature plasmacyte with the characteristic clear area near the eccentric nucleus. Finally, the term lymphocyte will refer to cells like *D* in figure 7.

The distribution of antibody-producing cells among different cell types is shown in table 5. In the micropipette experiments, 14 per cent of the cells of the lymphocytic series and 27 per cent of those of the plasmacytic series showed phage-inactivating activity. In one microdrop experiment comparable results were obtained. It should be noted that stem or blast cells also can produce antibody; the reticular cells examined, on the contrary, are too few to allow any conclusion as to their potentialities. These findings are important in that a direct measurement of antibody activity in a single cell has been correlated with cell morphological characteristics. Cells of both the plasmacytic and lymphocytic series contain and release antibody into the medium; this finding reconciles the divergent results obtained by the numerous workers (reviewed in (15, 16)) who have investigated this problem.

From the comparison of the proportion of antibody-producing cells in the whole cell population and in the plasmacytic series, it appears that a substantial increase in the proportion of antibody-producing cells can be obtained by selecting for these cells. No correlation has been observed between any morphological characteristics and amount of antibody detectable *in vitro*. This point has not been investigated in detail.

The cells which showed anti-T2,T5 activity in the micropipette experiments were all cells of the plasmacytic type. It is difficult, however, to evaluate at present the significance of this result because of the low number of observations. Finally, the production of two species of antibodies by a single cell was not found to be associated with the presence of two nuclei in the cell.

The experiments we have just described require a large number of different controls, directed toward answering the following questions. Is the phage inactivation observed *in vitro* in the presence of cells due to antibody? If phage inactivation is due to antibody, is there a synthesis of new antibody or a release of preformed antibody? In the case of double inactivation by a single cell, could this phenomenon be accounted for by a passive absorption of antibody by nonproducing cells, or by artifacts of the techniques?

Several lines of evidence indicate that the phage inactivation observed *in vitro* in the presence of cells is due to antibody. In the first place this inactivation is cell linked, as shown by three facts: (a) there is a linear relationship between K and cell concentration in a mass suspension; (b) the sum of the K's in the individual drops accounts roughly for the K of the mass suspension; and (c) the proportion of inactivating drops increases with the cell content of the drops according to expectations from a Poisson distribution of the antibody-producing cells. Additional evidence that antibody is responsible for phage inactivation *in vitro* is brought out by the fact that the inactivation is linked to active immunization and is not present after passive immunization, as we will discuss later, and by the fact that chick anti-rabbit γ-globulin serum

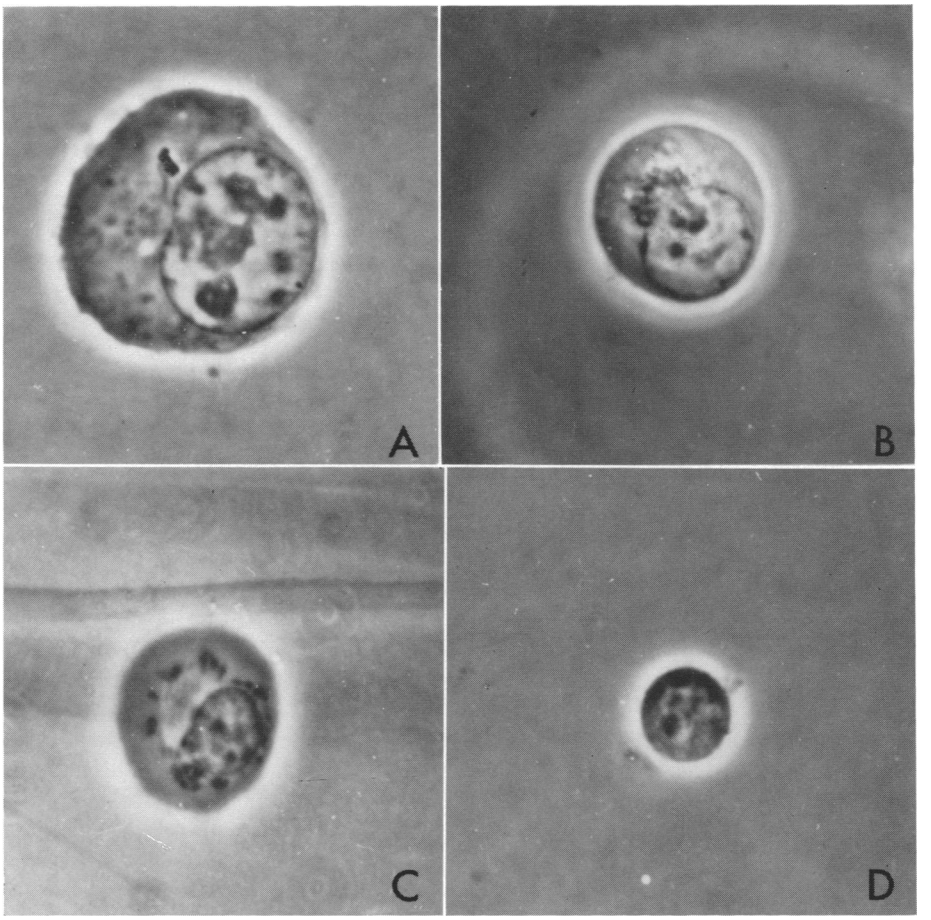

Figure 7. Typical lymph node cells photographed in living state in phase contrast. *A*, stem cell; *B*, immature plasma cell; *C*, mature plasma cell; *D*, small lymphocyte. Magnification, 1680×.

TABLE 5

Distribution of antibody-producing cells among different cell types

Experiments	Lymphocytic Series (*D*)	Plasmacytic Series (*B*, *C*)	Stem Cells (*A*)	Reticular Cells	Total
Micropipette					
No. cells tested	44	109	16	2	171
No. Ab producers	6	29	2	0	37
Per cent antibody producers	14	27	13	0	22
Microdrop					
No. cells tested	92	34	2	1	129
No. Ab producers	10	11	0	0	21
Per cent antibody producers	11	32	0	0	16

The letters in parentheses at the heads of the columns refer to cell types in figure 7.

protects phage against inactivation by cells as a result of neutralization of antiphage antibody.

As to the problem of whether an actual production of new antibody or a release of preformed antibody occurs *in vitro*, two pieces of evidence favor the first alternative. On the one hand, the cell suspensions incorporate C^{14}-labeled amino acids into γ-globulin; on the other

hand, the antibody which can be extracted from the cells before incubation is less than 10 per cent of the amount detectable in the supernatant after incubation.

In order to investigate the possibility of a passive taking up by some cells of antibody released by other cells, several experiments were performed in which cells were isolated from animals actively immunized against one strain of phage and passively against the other strain. For example, in one such experiment the animal was actively immunized against T2 and injected 24 hr prior to sacrifice with γ-globulin isolated from 800 ml of pooled strong anti-T5 sera. The KT2 of this animal's serum before this injection was 2100 and the KT5 was <0.01. At 25 min after the injection, the KT5 had increased to 653; 24 hr later, at the moment of sacrifice, the KT5 was 200. A total of 298 drops, containing an average of 23 cells per drop, were analyzed; 199 drops, that is 67 per cent, showed anti-T2 activity. The calculated proportion of anti-T2 cells was 5 per cent. By way of contrast, no drop showed anti-T5 activity and, therefore, the proportion of anti-T5 cells was <0.01 per cent. Similar results were obtained with cells taken from rabbits immunized actively against T5 and passively against T2. Within the limits of significance of these experiments, such findings exclude a passive absorption of antibodies as a way of interpreting the presence of bispecific antiphage-forming cells. Finally, as a control on the technique, a reconstruction experiment was made by mixing two cell populations derived from two different animals, each one actively immunized against only one phage. The failure to find any double inactivating single-cell drops (<0.4 per cent) with this mixed suspension leaves little doubt that double inactivation by a single cell could be simulated by errors in the scoring of the drops, in their recovery, in plating, or by other mistakes.

SUMMARY

The evidence presented favors the conclusion that one cell can synthesize antibodies to two distinct antigens. What are the implications of this result? The theories of antibody formation can be divided into two classes, depending upon the assumption they make regarding the range of the antibody-forming capacity of a single cell: (a) *restrictive* theories (17–19), which postulate either one, or at most, two antibody-forming potentialities in a single cell and (b) *unrestrictive* theories (20–23), which do not put *a priori* any restriction to the synthetic capacity of a given cell.

Our results do not support the theories (17, 18) which postulate only one antibody-forming potentiality per cell. Apart from this, the significance which can be attributed to the fact that one cell can make antibodies to two distinct antigens depends on the extrapolation one wants to make. If one considers two as the maximum number of antibody-forming potentialities in a given diploid cell, then antibody synthesis can be referred in the simplest case to some kind of genetic mechanism, with two genes per diploid set of chromosomes, each controlling the formation of a specific antibody. If one is willing to extrapolate from two to many, that is, to consider the antibody-forming capacity of a given cell to be as large as that of the animal, then antibody formation may be considered as a kind of unrestricted inductive process. If, finally, one extrapolates from 2 to 3 or any low number, then one can think of a genetic mechanism with more than two genes involved per diploid set of chromosomes, or with a polyploid set of chromosomes, or assume an inductive process with some kind of interference or competition between the antigenic stimuli. Further speculation along these lines would be unproductive without additional information.

The purpose of this paper has not been to speculate, but rather to illustrate a particular experimental approach to the problem of antibody synthesis. Populational analyses will not elucidate the mechanism of antibody synthesis at the chemical level, but they wlll give us the information necessary to decide in which direction we should go to understand better the nature of the antibody response.

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