PRIMARY STIMULATION AND MEASUREMENT OF ANTIBODY PRODUCTION TO SHEEP RED BLOOD CELLS IN VITRO*

By W. A. ROBINSON, M.D.,‡ J. MARBROOK, M.Sc.,§ and E. DIENER, Ph.D.

(*From The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia*)

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Recent papers have described primary antigenic stimulation and measurement of antibody formation in vitro (1, 2, 3). Ideally an in vitro system would (*a*) reproduce the in vivo immune response, (*b*) provide a much needed way of measuring the number and types of cells responsive to antigenic stimulation, (*c*) allow continued growth of and antibody production by stimulated cells. Unfortunately such a system is not yet available. Borrowing from a recently described method of bone marrow culture (4) and the hemolytic plaque assay system for cellular antibody production to sheep erythrocytes described by Jerne (5), we have designed a system with these ends in mind. In this system single cell suspensions of spleen cells from unimmunized mice can be primarily stimulated to produce antibody against sheep erythrocytes with the production of complement-dependent hemolytic plaques. The plaques formed gradually increase in size with continuing incubation and at the end of 3 days are much larger than those seen in the simple Jerne plaque assay system. Limited cellular division of primarily stimulated cells appears to occur and is necessary for the formation of sufficient antibody to lead to a plaque area. This system is quantitatively and qualitatively reliable, technically simple, and offers many technical and theoretical advantages over presently available in vivo techniques for the enumeration of cell numbers responsive to antigenic stimulation.

Technique

Normal 2-month-old male CBA mice were used as cell donors in all experiments.

Cultures were set up in 90 mm plastic Petri dishes. In an initial set of experiments kidney cell feeder layers in agar gel medium were used as a base layer as in previously described bone marrow cultures. The addition of mouse kidney cells to the base layers did not, however,

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§ Present address: Department of Cell Biology, University of Auckland, Auckland, New Zealand.

alter the number or size of plaques developing and in the experiments to be described these were not used. Base layers were prepared by mixing equal volumes of 1% agar (Bacto-Agar, Difco Laboratories, Inc., Detroit, Mich., previously boiled and cooled to 40°C) and double strength Eagle's minimum essential media (supplemented by 10% fetal calf serum, 10% trypticase soy broth, and 21 mg of L-serine and 110 mg of pyruvate per liter]. 5 ml aliquots of this were plated into Petri dishes and allowed to gel at room temperature. Plates were used on the same day as prepared.

Spleens were removed aseptically, the cells teased from the capsule and prepared as a single cell suspension in Eagle's medium. Appropriate concentrations were then added to the overlay medium prepared by mixing equal volumes of 0.8% agar and double strength Eagle's minimum essential medium (supplemented as above). To the overlay medium was added sheep erythrocytes (2×10^8 cells/ml) and sterile DEAE dextran (0.5 mg/ml). 2 ml aliquots of this mixture were then pipetted onto the previously prepared base layers. In the addition of the cell layer great care was taken to avoid bubbles and to keep the media warmed to 40°C while pipetting. Cooling results in clumping of sheep erythrocytes and bubbles lead to spurious plaques. After cooling at room temperature the plates were incubated at 37°C in a humidified incubator with a constant flow of 5% CO_2 in air. Plates were developed by the addition of 2 ml of complement as 1:5 fresh guinea pig serum.

Plaque counting and size measurements were done with a dissecting microscope at $\times$ 10 magnifications using an ocular micrometer. Plates were examined for bubbles or other imperfections prior to the addition of complement which might masquerade as plaques. These were marked by an ink spot on the bottom of the dish.

Initial experiments showed that the number and size of plaques did not increase after day 3 of incubation and that much lysis and breakdown of red blood cells occurred after this time. For this reason routine counts were done at day 3 of incubation.

In experiments using bone marrow and thymus cells these were obtained from spleen donor animals in the same experiment and prepared in the same way. Killed spleen cells were obtained from control pools and heated to 60°C for 30 min. Anti-mouse globulin was prepared in rabbits as previously described (6), inactivated by heating to 56°C for 30 min and absorbed with mouse spleen cells three times. In experiments using colchicine, actinomycin D, phytohemagglutinin, and anti-mouse globulin, these were added to the cell layer only at the time of plating in the concentrations shown in Table II.

For microscopic examination the centers of plaque areas were removed with a finely drawn Pasteur pipette and placed on marked glass slides. Cover slips were then gently lowered on the agar droplets and 0.25% orcein in 50% acetic acid floated under the cover slips for staining.

RESULTS

No plaques were visible until after the addition of complement. Control plates incubated with sheep erythrocytes without the addition of spleen cells yielded no plaque areas. On many plates small colonies of cells could be seen at day 3 of incubation similar in appearance to those described on bone marrow culture plates (4). On microscopic examination these were found to be composed largely of members of the granulocytic series in varying states of development and breakdown. These were not associated with plaque areas.

Fig. 1 shows the number of plaques found after varying times of incubation with 10×10^6 spleen cells. Eighteen plates which were developed with complement immediately after plating or within 2 hr had an average of 0.16 plaque areas per plate. This was taken as the background or normal level. Time points

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between 2 hr and 24 hr were not performed in this initial set of experiments. The number of plaques formed increased from plating until day 2 but did not significantly increase from day 2 to day 3. Culture and plaque counting beyond this time was not uniformly reliable because of much lysis and breakdown of red cells. While the number of plaques formed did not increase from day 2 to day 3, the size of the plaques formed did increase, as shown in Figs. 2 and 3, indicating continued antibody production and release during this time. The larger number and size of plaques at days 2 and 3 as compared to day 1 may

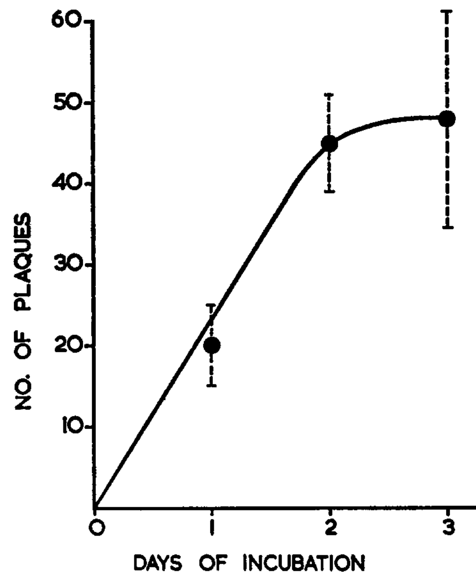

FIG. 1. Number of plaques formed at varying times of incubation with 10×10^6 spleen cells per plate. Bars represent standard deviations. Each point represents the mean of 10–20 plates.

represent the increasing recruitment of sensitive cells or, more likely, the fact that many potential plaque areas were too small to distinguish at day 1.

The number of plaques formed with increasing numbers of spleen cells is seen in Fig. 4. The linear relationship depicted suggests that each plaque area is formed originally from a single stimulated cell.

It should be noted that the figures plotted in Fig. 4 are the result of two experiments and that the number of plaques formed per 10^6 cells plated is somewhat higher than our usual value of about 4. Like the Jerne plaque assay system there are minor variations from day to day in the numbers of plaques seen, probably as the result of slight daily variations in cell dilutions, pH of the media, incubator conditions, and efficacy of complement. These are not significant when adequate controls are included in each experiment.

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Table I shows a comparison of the plaque-forming ability of spleen, killed spleen, bone marrow, and thymus cells from normal unstimulated animals. Of the cells tested only those from the spleen contained a significant number

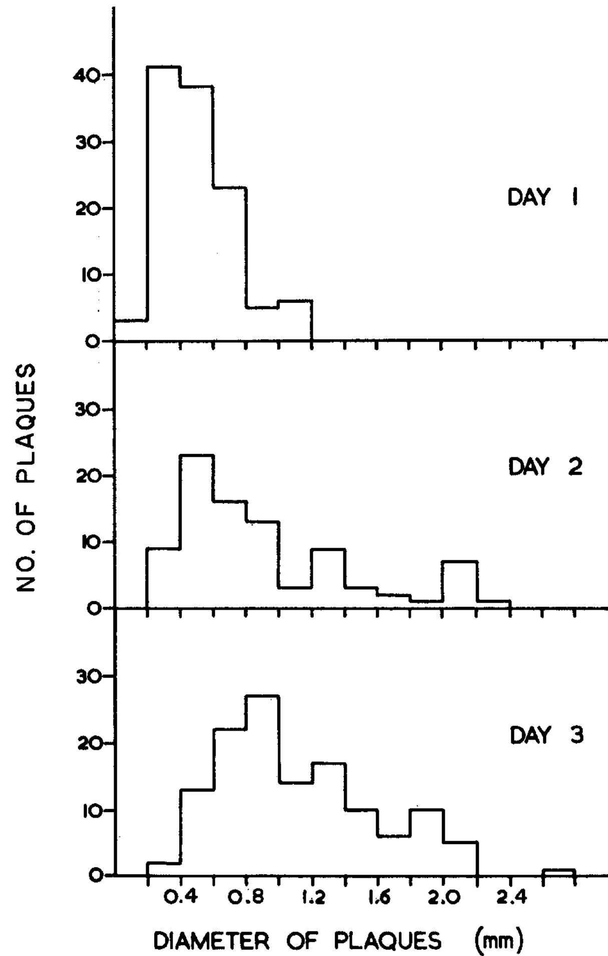

FIG. 2. Histogram of the distribution of plaque size on 1, 2, and 3 days of incubation.

of cells responsive to antigenic stimulation by sheep erythrocytes. The absence of hemolytic plaque formation in these experiments with varying kinds of cells indicates that plaque development is not the result of nonspecific cellular breakdown and lysis.

The results of the addition of rabbit anti-mouse globulin, colchicine, phytohemagglutin, and actinomycin D are seen in Table II. These results indicate

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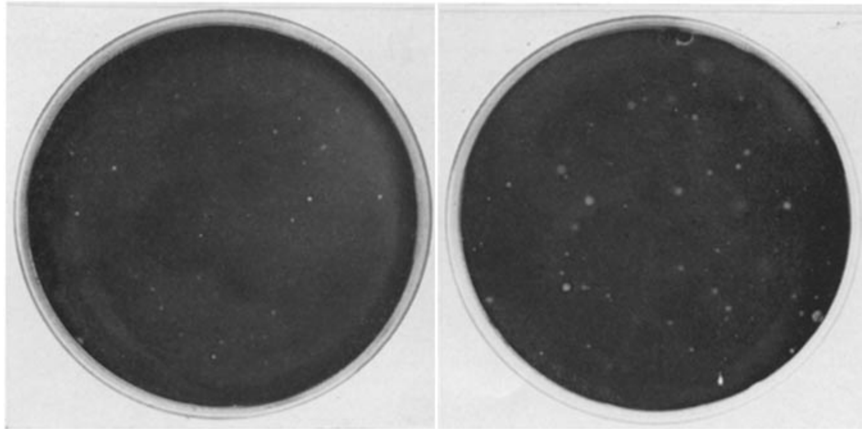

FIG. 3. Photographs of plaques developed with complement at day 2 (left) and day 3 (right) for comparison of plaque size. Stained with benzidine.

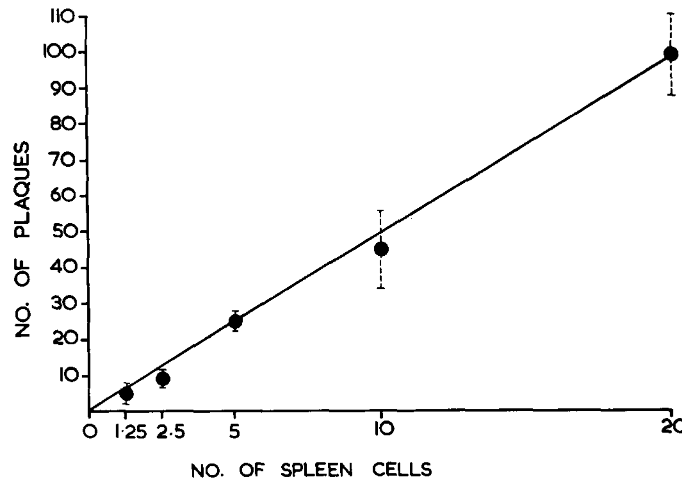

FIG. 4. The number of plaques formed with varying numbers of spleen cells. All plates developed and read at day 3 of incubation. Each point represents the mean of 10–20 plates. Bars represent standard deviations.

that cellular division, DNA-dependent RNA synthesis, and antibody release are all necessary for the formation of plaque areas. It should be noted that neither colchicine nor actinomycin D affected the number of background plaques and that phytohemagglutinin had no significant effect on plaque numbers, if anything causing a slight reduction in number.

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When viewed under the dissecting microscope, the centers of many plaque areas contain what appear to be small foci of cells which are presumed to be those responsible for the release of antibody and subsequent plaque formation. In some plaque areas, however, such recognizable foci could not be detected with certainty. Attempts to identify the actual plaque-forming cells by removal

TABLE I

Number of Plaques Developing after 3 days Incubation of Various Kinds of Cells with Sheep Erythrocytes

Cell type	Mean No. plaques/10 $\times$ 10^6 cells	No. plates	Range
(No cells, sheep erythrocytes only)	0	8	0
Normal spleen	44	7	38–53
Killed spleen	0.1	10	0–1
Bone marrow	0.3	10	0–2
Thymus	0.1	10	0–1

TABLE II

The Effect of Colchicine, Actinomycin D, Anti-Mouse Globulin, and Phytohemagglutinin on Plaque Formation by 10 $\times$ 10^6 Spleen Cells

	Mean No. plaques	No. plates	Range
Spleen cell control	37	7	25–51
Actinomycin D 2 μg/ml	2	10	0–5
Colchicine 2.5 μg/ml	2	9	1–4
Rabbit anti-mouse globulin			
0.25 ml/plate	0	6	0
0.10 ml/plate	0	6	0
Photohemagglutinin 10 μg/ml	24	8	16–36

of these areas from the agar with finely drawn Pasteur pipettes have been unsatisfactory to date because contamination by surrounding cells made identification of the actual plaque-forming cells unreliable. Similarly, to remove cells from plaque areas and replate them in the Jerne plaque assay system has, so far, been technically unsatisfactory and is currently being attempted with more refined techniques.

DISCUSSION

The major importance of the present work is that this system offers an in vitro method for primary stimulation and measurement of cells responsive to

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antigenic stimulation by sheep erythrocytes in whole spleen cell populations. The number of such antigen-sensitive cells in the mouse spleen has been variously estimated between 50 and 5000 by a number of workers using different techniques. Makinodan and Albright (7), studying antibody titers in diffusion chambers, estimated that 1 in 10^7 spleen cells from nonimmunized animals was a potentially antigen responsive cell. This figure seems somewhat low in view of later studies and more direct means of measurement. Bussard (2), using a micromethod technique in which spleen cells were incubated with sheep erythrocytes in carboxymethyl cellulose on microscope slides, gave an estimate of between 1 and 13 antigen-sensitive cells per 10^6, or 100 to 1300 per whole spleen assuming an average mouse spleen contains 10^8 nucleated cells. In Bussard's experiments this number could be greatly increased by incubation of spleen with peritoneal cells. Playfair, Papermaster, and Cole (8) found 5000 cells in a whole mouse spleen which were responsive to antigenic stimulation by sheep erythrocytes. Their estimates were made using a hemolytic focus assay system in irradiated mice. Claman et al. (9) using the same technique estimated the number at 700. Kennedy et al. (10, 11), using a similar hemolytic focus assay system and the technique of limiting dilution, set the number between 500 and 3000 antigen-sensitive cells per whole mouse spleen. In a more recent report Miller and Mitchell (12), using the assay method described by Kennedy (10, 11), found 15 antigen-sensitive cells per million nucleated spleen cells or a total of 1500 per whole mouse spleen.

The figure for the number of cells in the mouse spleen responsive to antigenic stimulation by sheep erythrocytes obtained in the present work is in reasonable agreement with the estimates of the last three authors using in vivo techniques. Using a mean value of 4.4 plaque areas per million cells plated (Table I) our estimate would be about 440 antigen-sensitive cells per whole 2-month-old CBA mouse spleen. This figure is somewhat lower than what would seem to be the most reliable in vivo estimates. It may be that the present figure is a closer estimate of the actual figure since it is the result of direct measurement and does not rely on calculations from uncertain cell dilution and multiplication factors.

Many of the other data presented here are in close agreement with those obtained by other workers using in vivo methods. Both our experiments and those of Kennedy (10, 11) and Claman (9) have failed to show the presence of significant numbers of antigen-sensitive cells in the bone marrow and thymus when used alone as a cell source. The latter workers have, however, reported that combinations of these two are effective in producing a population responsive to antigenic stimulation by sheep erythrocytes (9). Although no cells from the thymus or bone marrow were found which produced hemolytic plaques, the method described was devised to measure such cells in the spleen and details of the possible dependence of plaque-forming ability on more than one cell type in the spleen have not yet been fully investigated.

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Like Harris and Littleton (13) studying the response of rabbit spleen cells to sheep erythrocytes, we failed to note any effect of phytohemagglutinin on the number of cells responsive to stimulation.

The noted effect of actinomycin D in the present experiments indicates that *de novo* synthesis of protein is required for the production of a hemolytic area.

As there was no plaque formation after the addition of colchicine to the medium it would appear in the system described, as well as in vivo (10, 11) that cellular division is necessary for the production of sufficient antibody to produce a hemolytic plaque. The observed lag phase with increasing numbers of plaques until 2 days of incubation is consistent with this conclusion.

It is of considerable interest that the number of background plaques was not affected by the addition of either actinomycin D or colchicine at the concentrations used. As rabbit anti-mouse globulin does suppress background plaque formation two conclusions are prompted. The first conclusion is that it is not necessary for the appropriate cells to divide before the events leading to background plaque formation; secondly, there must exist in normal spleen cells prior to contact with sheep erythrocytes a specific messenger RNA for anti-sheep red blood cell antibody. It would be necessary to conduct experiments with radioactive precursors of proteins and nucleic acids to fully substantiate these conclusions.

Also to be added to the evidence of cellular division in the present system is the frequent, though not invariable, finding of small foci of what are presumed to be the antibody-producing cells in the center of plaque areas. After 3 days of incubation some foci are probably still too small to be recognized clearly. By removing the more obvious foci, counting the number of cells present and the number of those producing antibody by replating dispersed foci in a Jerne plaque system, one might arrive at the actual rate of cellular division of antibody-producing cells at various times. Preliminary experiments in an attempt to do this have begun. These have been technically difficult because of contamination of surrounding cells when removing plaque areas and difficulty in dispersing the agar-containing foci in order to get a single cell suspension for replating. More refined microtechniques are now being developed.

Despite evidence of cellular growth and division of primarily stimulated antibody producing cells, it is likely that the present system will not answer the vexing problem of long term clonal growth of such cells. The number of both target and sheep erythrocytes that have to be plated to produce countable, quantitatively reliable numbers of plaque areas is not compatible with sustained growth and division beyond a limited time. It is of considerable interest that kidney cell feeder layers did not alter the number of plaques formed. It may be that the large number of cells plated served as their own feeder layer or that other cells may be found which supply a stimulus not found in kidney cells and are therefore more effective as feeder layers. It would

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seem, however, that the greatest deterrent to growth in the present system is not the lack of available factors but the great number of toxic products released from dying cells. The base layer used probably serves a twofold function as a means of dialyzing away some of this material and a source of nutrient supply.

The present system should be adaptable to the study of other antigenic materials, some not as toxic in culture as sheep erythrocytes. It should provide a simplified alternative means for the present in vivo techniques of measuring numbers of potentially antigen-responsive cells in various populations, provide a more direct means of looking at cellular interaction in the immune response, and may be a major step toward the clonal growth of antibody-producing cells.

SUMMARY

A method for the primary stimulation and measurement of antibody production to sheep red blood cells in vitro has been described. In this system when mouse spleen cells are incubated with sheep erythrocytes large complement-dependent plaques are formed. The number and size of plaques increases from day 1 to day 3 of incubation with an average of 4.4 plaque areas per 1×10^6 cells plated at day 3. There is a linear relationship between the number of spleen cells plated and the number of plaques formed. Plaque formation is inhibited by colchicine, actinomycin D, and rabbit anti-mouse globulin. This system offers a possible means for the direct in vitro measurement of the number of cells in a population susceptible to antigenic stimulation by sheep erythrocytes.

BIBLIOGRAPHY

1. Tao, T. W., and J. W. Uhr. 1966. Primary-type antibody response *in vitro*. *Science* **151**:1096.
2. Bussard, A. E. 1966. Antibody formation in non-immune mouse peritoneal cells after incubation in gum containing antigen. *Science* **153**:887.
3. Mishell, R. I., and R. W. Dutton. 1966. Immunization of normal spleen cell suspensions *in vitro*. *Science* **153**:1004.
4. Bradley, T. R., and D. Metcalf. 1966. The growth of mouse bone marrow cells *in vitro*. *Australian J. Biol. Med.* **44**:287.
5. Jerne, N. K., and A. A. Nordin. 1963. Plaque formation in agar by single antibody cells. *Science* **140**:405.
6. Fahey, J. L., and C. McLaughlin. 1963. Preparation of antisera specific for 6.6 S γ-globulins, β2A-globulins, γ'-macroglobulins, and for Type I and II common γ-globulin determinants. *J. Immunol.* **91**:484.
7. Makinodan, T., and J. F. Albright. 1962. Cellular variation during the immune response: One possible model of cellular differentiation. *J. Cellular Comp. Physiol.* **60** (Supp. 1):129.

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8. Playfair, J. H. L., B. W. Papermaster, and L. J. Cole. 1965. Focal antibody production by transferred spleen cells in irradiated mice. *Science* **149**:998.
9. Claman, H. N., E. A. Chaperon, and R. F. Triplett. 1966. Thymus-marrow cell combinations. Synergism in antibody production. *Proc. Soc. Exptl. Biol. Med.* **122**:1167.
10. Kennedy, J. C., J. E. Till, L. Siminovitch, and E. A. McCullough. 1966. The proliferative capacity of antigen-sensitive precursors of hemolytic plaque-forming cells. *J. Immunol.* **96**:973.
11. Kennedy, J. C., L. Siminovitch, J. E. Till, and E. A. McCullough. 1965. A transplantation assay for mouse cells responsive to antigenic stimulation by sheep erythrocytes. *Proc. Soc. Exptl. Biol. Med.* **120**:868.
12. Miller, J., and G. Mitchell. 1967. The cellular basis of the immunological defects in thymectomized mice. II. The immunologic competence of cells in the thoracic duct lymph of mice thymectomized at birth. *Nature* **214**:994.
13. Harris, G., and R. J. Littleton. 1966. The effects of antigens and phytohemagglutinin on rabbit spleen cell suspensions. *J. Exptl. Med.* **124**:621.

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