Plaque Formation in Agar by Single Antibody-Producing Cells

Abstract. *Distinct plaques, each of which is due to the release of hemolysin by a single antibody-forming cell, are revealed by complement after incubation, in an agar layer, of a mixture of sheep red cells and lymphoid cells from a rabbit immunized with sheep red cells.*

We have developed a simple technique for scoring individual antibody-forming cells among a mixed cell population. The following experimental example describes the procedure.

From a rabbit that had received three injections, each of 5 times 10^9 sheep red cell stromata, in the footpads during the preceding three weeks, a popliteal lymph node was removed and its contents of cells teased out into tissue culture medium containing no serum (*1*). Microscopic examination of the cell suspension obtained after washing by three centrifugations and resuspensions in this medium in the cold showed the presence of non-aggregated lymphoid cells of various types. One million of these cells in 0.1 ml as well as 200 million sheep red cells in 0.1 ml were added to 2 ml of a 0.7-percent Difco-agar solution in the culture medium that was kept fluid at 45°C, and the mixture was poured onto a supporting 1.4-percent agar bottom layer in a petri dish so as to form a thin, semisolid top layer. After incubation at 37°C for 1 hour this layer was covered with 1.5 ml of complement (guinea pig serum 1 : 5). Further incubation for 15 minutes revealed about 100 clear plaques of about 0.25 mm diameter that stood out sharply against the uniformly red background.

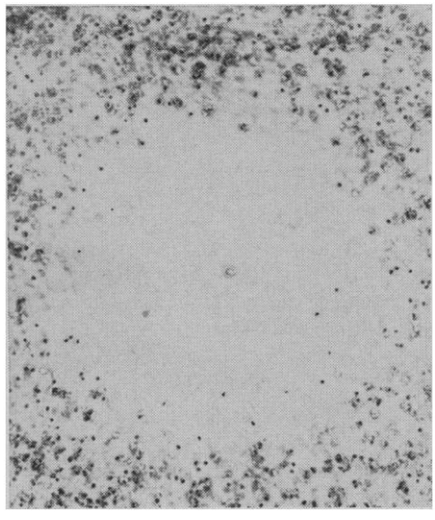

Fig. 1. Rabbit lymph node cell producing a plaque.

Experiments of this type with rabbit lymph node cells as well as with mouse spleen cells have shown that the number of plaques obtained is proportional to the number of lymphoid cells plated. This suggests that each plaque is due to the activity of an individual cell. Microscopically, a plaque shows up as a circular hemolytic clearance in a field of closely scattered red cells, and in the center of most plaques a lymphoid cell is seen which presumably is the cell that released the sensitizing antibody (Fig. 1). Larger plaque sizes are obtained by using a lower red cell concentration, though this diminishes the color contrast. Plaque formation did not occur in the presence of 0.01 molar potassium cyanide.

By using red cells coated with other antigenic determinants, the technique may be extended to other antigen-antibody systems, whereas multiple antibody production by individual cells could be studied by using mixed red cells. We are now investigating the question of the efficiency of plating.

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Reference

1. G. A. Fischer, *Ann. N.Y. Acad. Sci.* **76**(3), 673 (1958).

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Antibody Quality after Sequential Immunization with Related Antigens

Abstract. *The response to the second antigen of a sequence of two related antigens appears to consist of two qualitatively different antibody populations. Antibodies specific for the second antigen are of primary-response quality while the antibodies which cross-react with the first antigen are of secondary-response quality.*

The substantial body of information demonstrating qualitative differences between antibodies produced after a first and second injection of the same antigen (*1*, *2*) makes it necessary to re-evaluate interpretations of antibody responses based solely on quantitative data. One such situation is the response to sequential immunization with two related antigens. The conclusions based on past studies range from (i) anamnestic (secondary) responses to both antigens (*3*) to (ii) a response directed mainly against the first antigen of the sequence (*4*) to (iii) a response directed mainly against the cross-reacting determinants of the two antigens along with a primary response specific for the second antigen and a small amount of antibody specific for the first antigen (*5*). We have been able to clarify this problem partially by studying antibody quality, as measured by the rate of dissociation of labeled antigen from antigen-antibody complexes.

White Rock male chickens, 10 weeks old, obtained from a commercial breeder were injected with 40 mg of one antigen, either crystalline bovine serum albumin (BSA) or human serum albumin (HSA), followed 45 days later by 40 mg of the same or the other antigen. In the chicken approximately 10 percent of the antibodies produced against either antigen can be precipitated with the other antigen (*5*). Serum samples were obtained 6, 8, and 15 days after each injection as well as 43 days after the first injection. In order to obtain specific antibodies, the cross-reacting activity was absorbed out by incubating the serum with a large excess of the appropriate antigen for several days. Our technique is a modification of that described previously by Farr (*2*) and Talmage (*6*). Serum which had been diluted to bind 20 to

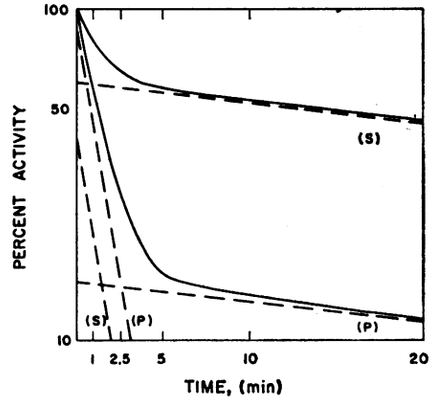

Fig. 1. Dissociation curves typical of antisera obtained 8 days after one (*P*) and two (*S*) injections of either BSA or HSA. Dotted lines represent the two components of each curve.

Table 1. Percentage (mean ± standard deviation) of slowly dissociating component in anti-BSA sera of various types 6, 8, and 15 days after the last injection. Means were derived from five to eight individual animals. The injection sequence is indicated by italic type; sera indicated were absorbed with HSA.

6 days	8 days	15 days
BSA, unabsorbed		
14.9 ± 4.8	18.4 ± 4.8	20.1 ± 4.0
BSA-BSA, unabsorbed		
55.7 ± 9.0	58.1 ± 9.4	58.8 ± 9.8
*BSA-BSA, absorbed**		
53.9 ± 13.4†	57.0 ± 9.9†	Not done
HSA-BSA, unabsorbed		
44.1 ± 7.5‡	45.6 ± 12.0‡	50.3 ± 9.9†
HSA-BSA, absorbed		
15.7 ± 5.4§	17.3 ± 5.5§	26.3 ± 6.8§

* This control indicates that absorption did not affect the results nonspecifically. Statistical analysis (99 percent confidence interval) was carried out by the method of Duncan (*8*). † Not different from secondary value. ‡ Different from both primary and secondary values. § Not different from primary value.

45 percent of an I^{131}-labeled albumin (*7*) preparation (0.16 μg of nitrogen per milliliter) was mixed with the albumin solution and incubated at 37°C. After the mixture had reached equilibrium (30 minutes was sufficient), a 2000-fold excess of unlabeled albumin was added. The amount of I^{131}-albumin remaining bound to antibody was determined at intervals of 1, 2.5, 5, 10, and 20 minutes by stopping the reaction with ammonium sulfate added to 50-percent saturation. This concentration of ammonium sulfate precipitates albumin-antibody complexes and leaves unbound albumin in the supernatant. The activity of the precipitates was determined in a well-type scintillation counter, and appropriate corrections were made for nonspecific binding to normal serum. The results were plotted on a semilogarithmic scale as the percentage of the bound antigen that had been formed in a sample taken before the addition of excess unlabeled antigen (Fig. 1).

All antisera, regardless of origin, yield dissociation curves (Fig. 1) which can be treated as summations of two independently dissociating components. By applying the methods used in calculating half-lives of mixtures of two radioactive isotopes with different decay constants, mean half-lives of approximately 1 minute for the rapidly dissociating component and 50 minutes for the slowly dissociating component were calculated for all sera tested. Statistical analysis (*8*) revealed no differences in half-lives of either component in any type of antiserum; therefore the only difference among the various types of antisera was change in the ratio of the two components. Thus we were able to express our results as the percentage composition of either component in any given antiserum. All results are percentages of the slowly dissociating component present at zero time. All anti-BSA determinations are given in Table 1; anti-HSA responses were only analyzed graphically because fewer animals were involved. No obvious differences in response pattern were observed between the two antigens.

The data show that antibodies *specific* for the second antigen of a sequence of related antigens are of primary quality and that no maturation of the immune response to the second antigen has occurred. Further, the increased percentage of the slowly dissociating component in the total response was demonstrated, by the absorption technique, to be a result of antibodies of secondary-response quality produced against the cross-reacting determinants of the two antigens. Based on the results with chemically defined cross-reacting antigens (*4*) we expect these antibodies to have their greatest affinity for the first antigen. Little can be said about the reappearance of antibodies *specific* for the first antigen (*3*, *5*) because just before the second antigen injections antibodies of approximately secondary quality were found in the serum (three samples averaged 48 percent slow component).

Considering the overall result, there is an interesting dichotomy in response to cross-reacting and unique determinants of the second antigen; the unique (specific) determinants stimulate primary quality antibody while the cross-reacting determinants stimulate secondary quality antibody (*9*).

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References and Notes

1. N. K. Jerne, *Acta Pathol. Microbiol. Scand. Suppl. No. 87* (195 → D. C. Bauer and A. B. Stavitsky, *Proc. Natl. Acad. Sci. U.S.* **47**, 1667 (1961).
2. R. S. Farr, *J. Infect. Diseases* **103**, 239 (1958).
3. F. J. Dixon and P. H. Maurer, *J. Immunol.* **74**, 418 (1955).
4. J. M. Dubert, thesis, University of Paris (Chiron, Paris, 1959); ———, *Compt. Rend.* **243**, 1939 (1956).
5. R. V. Gilden, *Immunology* **6**, 30 (1963).
6. D. W. Talmage, *J. Infect. Diseases* **107**, 115 (1960).
7. Proteins were labeled with 1 to 2 atoms of iodine per molecule. Such preparations were 98 percent precipitable with excess antibody.
8. D. Duncan, *Biometrics* **13**, 164 (1957).
9. Supported by public health fellowship EF 14893 from the National Institute of Allergy and Infectious Diseases to S. Tokuda, by public health fellowship GPD-10897 from the Division of General Medical Sciences to R. V. Gilden, by Atomic Energy grant AT(04-3)-41 to Prof. R. D. Owen, and also by the Max C. Fleischmann Foundation of Nevada.
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Germination of Melampyrum lineare: Interrelated Effects of Afterripening and Gibberellic Acid

Abstract. *Seed germination of* Melampyrum lineare *proceeds only after activation followed by an extended period of chilling. Up to one-third of seeds can be activated by storage at room temperature, while all seeds are activated if treated with gibberellic acid. Chilling before activation is ineffective.*

Melampyrum lineare Desr. is an annual, chlorophyllous angiosperm which forms root connections with various other vascular plant species in its habitat. Studies of the life cycle and host-parasite relationship have been reported elsewhere (*1*), and indicate that for normal growth *M. lineare* must form connections with other species. In attempting to germinate *Melampyrum* seeds we have encountered a complex germination pattern, involving an apparently unreported relation between gibberellic acid and afterripening.

Various batches of seed were collected from natural populations growing in a jack pine forest near Grayling, Michigan, during the late summer and fall of 1960, 1961, and 1962. After harvesting, the seeds remained viable [as tested with tetrazolium chloride (*2*)] if stored turgid on moist blotters, either at room temperature (19° to 24°C) or in the cold (about 3°C). Seeds were found to require a period of chilling (moist storage at 3° ± 1°C) for germination, and remained dormant if stored at room temperature. This dormancy is complex, involving both a radicle- and a separate epicotyl-dormancy. Longer cold treatment is necessary to overcome epicotyl dormancy than to cause the radicle to emerge. In the present report "germination" is