APPLICATION OF A LOCALIZED HEMOLYSIN REACTION FOR SPECIFIC DETECTION OF INDIVIDUAL ANTIBODY-FORMING CELLS

By JOSEPH S. INGRAHAM, Ph.D., and ALAIN BUSSARD Ph.D.

(From the Department of Microbiology, Indiana University School of Medicine, Indianapolis, and the Laboratoire d'Immunologie Celludaire, Institut Pasteur, Paris, France)

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Interpretation of the observed kinetics of serum antibody formation and the heterogeneity of antibody molecules would be assisted considerably if it were possible to distinguish those features which result from intrinsic characteristics of the cellular antibody-forming system and those which reflect simply the heterogeneity of the cellular population. Some of the advantages to be gained from studying antibody formation by individual cells have been discussed by Attardi *et al.* (1).

A number of studies have now been reported in which cells were isolated in microdrops and analyzed individually for antibody formation (2). However, the technical requirements of this method appear to make it impractical for the analysis of antibody formation during the early phase of an immune response or in other situations where the proportion of antibody-forming cells is a very small fraction of the total cell population. The investigation of questions involving the induction of antibody formation and the population dynamics of antibody-forming cells requires a method which will allow identification of individual antibody-forming cells in large numbers without isolation and analysis individually in microdroplets. It has been observed that when suspensions of lymphoid cells from rabbits immunized with sheep erythrocytes are mixed with sheep erythrocytes and complement in culture medium thickened by the incorporation of a gum and spread on microscope slides under sealed coverslips, localized hemolysis occurs around a small proportion of cells within 15 to 20 minutes at 37°C. This phenomenon is the basis for a method which makes it possible to identify cells releasing (and probably synthesizing) antibody, to distinguish cells with various levels of activity, and to observe individual cells by high power phase contrast microscopy during the period of antibody release (3).[1]

The purpose of this paper is to describe this method as we have developed it up to the present and to report some experimental applications.

[1] N. K. Jerne and A. A. Nordin have independently developed a similar technique using localized hemolysis but with agar as the thickening agent (4). We wish to thank Dr. Jerne for sending to us the manuscript of a paper describing this method in greater detail (5).

Materials and Methods

Tissue Cells.—Suspensions of lymphoid cells from the popliteal nodes of normal or immunized rabbits were used in most of our experiments. Rabbits were sacrificed by exsanguination from the carotid artery. At autopsy each node was chilled immediately in iced saline, trimmed free of fat, and transferred to a tared beaker kept on ice and containing 5 ml of tissue culture medium with 30 per cent of normal rabbit serum (NRS). The node was incised in several directions with a scalpel, and cells were teased into the medium. After removing a first crop of cells, the remaining tissue was minced with scissors and extracted with an additional 5 ml of medium. The concentration of cells was determined by counting in a hemocytometer, and in some experiments the per cent of viable cells was estimated by using resistance to staining with trypan blue as a criterium for viability (6). Aliquots of this suspension were centrifuged at 300 to 400 RPM for 5 minutes in a table model centrifuge in the cold and resuspended to 0.2 ml in a mixture of equal parts of bovine amniotic fluid (BAF) and NRS.

Immunizations.—Rabbits were immunized by injecting 0.2 ml of washed packed sheep erythrocytes (rbc) into the left foot-pad every other day for five injections. Three weeks or longer after the last injection, they were given a single similar reinjection and sacrificed on the 3rd day. Some cases where different schedules of immunization were used will be described under the individual experiments.

Culture Medium.—Medium 1066 of Parker *et al.* (7) was used in our early experiments, later mixture 199 of Morgan *et al.* (8) was used. For experiments with metabolic inhibitors and persistence of antibody formation *in vitro* the basal medium of Eagle (9) was prepared by adding concentrated solutions of glutamine, amino acids, and vitamins to the balanced salt mixture of Earle.[2] All appeared to be satisfactory for our work, although we did not make any controlled comparison of the results with these various preparations.

The thickened culture medium containing sheep rbc for use in detection of antibody-forming cells was prepared by homogenizing 0.6 gm of carboxymethoxycellulose[3] with 24 ml of medium 1066 for 5 minutes at 90 v in a virtis 45 blender. Two mg of streptomycin and 4000 units of penicillin were added in 0.5 ml of phosphate-buffered saline (PBS, which consisted of 0.85 per cent sodium chloride in 0.005 M sodium phosphate pH 7.4), the mixture was homogenized a further 10 seconds, and then chilled. The pH was adjusted to 7.4 with 5 per cent CO_2-air mixture in the presence of phenol red as an internal indicator. Cells from sheep blood collected in Alsevers' solution (10) were washed three times with about 20 volumes of PBS containing 0.5 per cent glucose and 0.2 per cent bovine serum albumin (BSA). Ten gm of the thickened medium was mixed with 1.4 ml of a 50 per cent suspension of the washed sheep rbc and homogenized by a single excursion of a teflon pestle turning at a moderate speed in a smooth surface glass homogenizer. This suspension could be stored in the refrigerator for 4 or 5 days if the sheep rbc were no more than 2 or 3 days old when it was first prepared.

Assay Procedure.—Precleaned microscope slides 75 × 100 mm were prepared for use by pasting four narrow strips of gummed label paper (approximately 0.1 mm thick) across them to make two rectangular areas 18 to 20 mm wide.

The day before an experiment 0.7 gm portions of the thickened rbc medium were weighed into 12 × 75 mm tubes which were closed with rubber stoppers and stored in the refrigerator. The thickened medium was handled conveniently by drawing it into coarse cotton-plugged

[2] The synthetic culture media used were obtained from Microbiological Associates, Bethesda.

[3] High Viscosity cellulose gum type 70H of Hercules Powder Co., Wilmington. The work reported in this paper was done with a sample of lot No. 4747 obtained in 1949. The material currently available is designated "7HP." This appears to be very similar to the older sample, but may be of slightly higher viscosity.

capillary pipettes and then expelling it by pushing the cotton plug down with a glass rod. To prepared large numbers of samples the mixture could be handled with a 10 ml hypodermic syringe fitted with a 4 inch, 14 gauge cannula. At the time of the experiment, 0.1 ml of guinea pig complement absorbed with sheep red cells was added to each tube of medium (this mixture will be referred to as the "indicator system") followed by 0.2 ml of tissue cell suspension in BAF plus NRS as described above. The ingredients were mixed thoroughly with a glass rod and then centrifuged at 500 RPM for 1 minute to eliminate bubbles. This centrifugation did not appear to cause any appreciable sedimentation of the cells in the thickened medium. The layer of foam was pushed to one side with a slender, flat-end spatula, and a sample relatively free of bubbles was drawn from the bottom of the tube into a 0.1 ml graduated pipette with the aid of a tight fitting heavy rubber dropper bulb. Samples of 0.03 ml were placed in the center of the rectangular area on the prepared slide and covered immediately with a 22 mm square No. 2 coverslip (0.17 to 0.24 mm thick). The coverslip was pressed down gently to rest on the paper strips thereby spreading the gum into a disc about 18 mm in diameter (Fig. 1). The preparation was then sealed around the edges by applying melted vaseline with a small brush and was incubated at 37°C. For more critical examination of the cells with the highest power immersion objectives the paper strips were omitted, thin coverslips were used, and the sample was reduced to 0.005 ml or less. With an active preparation of cells incubated on a warm stage discrete holes of hemolysis could sometimes be detected within 5 or 10 minutes and were usually visible to the naked eye within 20 to 30 minutes. By the end of 1 to 2 hours these areas had reached about one-half or more of the final diameter observed after incubation for an additional 18 hours. The slides could then be kept without gross alteration for several days at room temperature.

To verify the activity and specificity of the indicator system and for experiments to test the activity with various rbc and mixtures of rbc, slides were often prepared to which small pieces of filter paper containing antiserum had been added. Strips of filter paper 1 x 5 cm were soaked with 0.01 ml of serum per cm^2, dried in the refrigerator, and then cut into squares or triangles of 0.5 to 1 mm on the side. Three or four of these pieces containing various antiserums or normal serum could be placed on a slide so that the indicator system would flow out and surround them when the coverslip was applied. The gross appearance of this preparation is seen in Fig. 1.

Considerable "drainage error" is to be expected in delivery of this indicator system from a pipette. Furthermore, there are always some bubbles present. For the nominal 0.03 ml aliquots the weight of material actually placed on the slides was found to be from 21 to 23 mg with 0.1 ml graduated pipettes and from 22 to 24 mg with 0.2 ml pipettes. Therefore, cell counts have been multiplied by 1.35 to correct for the systematic sampling error.

Preparation of Sheep Red Cells Coupled with Antigens or Haptens.—Sheep cells were coupled with bovine gamma globulin (BGG) or bovine serum albumin (BSA) using the bisdiazobenzidine (BDB) method essentially as described by Stavitsky and Arquilla (11). We mixed 1 ml of 50 per cent washed sheep red cells with 16 ml of PBS containing 0.5 per cent dextrose and 20 ml of 0.1 per cent BGG in PBS and then added 10 ml of neutralized BDB reagent in the cold. After 30 minutes in ice with occasional agitation, the cells were centrifuged off, washed twice with 40 ml PBS containing 0.5 per cent dextrose and 1 per cent NRS, and then mixed with thickened culture medium as for normal red cells. Human serum albumin was attached to sheep cells in a similar manner. Red cells were coupled with sulfanilic acid as described by Ingraham (12).

EXPERIMENTAL

Technique and Controls of the Method.—Up to the present, we have been successful in observing the release of antibody by individual cells only with

the sheep rbc–guinea pig complement–rabbit anti-sheep rbc system and in a few experiments in which pigeon rbc were substituted for the sheep rbc. We shall describe later our attempts to extend this method to other rbc, to non-cellular antigens, and to mixtures of rbc.

Positive reactions were usually apparent within 30 minutes or less although occasionally they required 1 to 2 hours to develop. We have always incubated the slides for 4 to 6 hours, and usually overnight, before making final readings and counts. When observed with a 40 power phase contrast objective a fully developed positive reaction had a typical large, round lymphoid cell at the center surrounded symmetrically by an area of red cell ghosts and often one or more other lymphoid cells depending on their total concentration in the assay medium (Fig. 3 *D*). There were several kinds of faults or artifacts in the medium which had to be distinguished from positive reactions. Bubbles were quite obvious initially, but in time they were often absorbed into the medium leaving a small clump of cells surrounded by a void (Figs. 3 *A* and 3 *B*). On casual examination these areas could be confused with positive reactions. Discontinuities in the indicator system where the rbc were almost completely absent or present in very low concentration were apparently caused by small pieces of undissolved gum (Fig. 3 *B*). They were often irregular in shape rather than round and did not contain ghosts. Occasionally atypical areas of lysis were seen where the red cell ghosts were represented by small misshapen asymmetric fragments rather than by fairly uniform intact discs as in the typical reaction. These areas were also often irregular in shape, and it was usually impossible to find a typical intact lymphoid cell in the center. Sometimes they may have resulted from contamination of the slide or coverslip. In two experiments there was apparently contamination with particles of dust carrying some lytic agent. These areas were round and contained intact ghosts, but they had acellular particles of various size, shape, and appearance at the center. All of these faults occurred in slides with heat-inactivated complement as well as with active complement.

All preparations were evaluated and counted as a routine using a 2.5 power Zeiss planachromat objective with a phase contrast condensor slightly out of correct position to give somewhat of a darkfield effect. With a total magnification of 40 the holes left by bubbles or discontinuities were readily distinguished from the lysed areas around the active cells (Figs. 3 *A* and 3 *B*). For more critical examination to identify atypical lytic reactions a 40 power phase contrast objective was used. Non-specific lytic particles were not common and were seen in significant numbers only in the two experiments mentioned above. These were done at a time when construction was underway in the adjacent laboratories. Atypical lytic reactions were a significant fraction of the total only with preparations of low activity obtained from normal animals or at short times after injection. These were most common with cells from the

spleen. With such preparations all areas which appeared to be typical specific lysis under low power were verified under high power phase contrast. In all experiments a sampling of the positive reactions was also evaluated in this way. The appearance of a small area of lysis in a thin preparation with the × 2.5 planachromat as described above and with × 10 and × 40 phase objectives is shown in Figs. 3 *B* to 3 *D*. The appearance of the cell at the center of the reaction of Fig. 3 *D* is not typical since it was undergoing extensive morphological alteration. Shortly before the picture was taken, this cell resembled the smaller cell appearing at 7 o'clock.

A brief account of this method has already been published (3). It was found that with four rabbits immunized as described under Materials and Methods the number of actve cells per million lymph node cells ranged from 118 to 164 (or 159 to 220 corrected for sampling error). In one experiment, 5 serial 2-fold dilutions of the suspension of lymphoid cells for assay were prepared. The number of active cells observed per million was constant within ± 10 per cent (187 per 10^6) for the first three dilutions (from 100 × 10^6 to 12 × 10^6 total cells per ml of assay mixture) and within ±20 per cent over the whole 30-fold range of dilutions. Heating the tissue cells at 56°C for 30 minutes, inactivation of the complement at 56°C for 30 minutes, or addition of sodium citrate (final concentration 0.01 M) reduced the number of active cells found by 90 to 98 per cent. Furthermore, when rabbits were injected in only one foot-pad, the proportion of active cells from the uninjected node was only 1 to 5 per cent of that from the immunized node of the same animal. When veronal buffer was substituted for medium 1066 in the indicator system, the proportion of active cells was reduced by 70 per cent.

Metabolic Inhibitors.—As an approach to the question whether antibody is being actively synthesized or simply released from storage in the cells, we have observed the effect of several metabolic inhibitors on cell viability and plaque formation.

Rabbits were immunized and suspensions of lymph node cells prepared as described in the methods. In the first experiment (rabbit 5-31), 0.01 ml of solutions of the inhibitors in water or in 95 per cent ethanol were added to 1 ml of the indicator system containing 8 × 10^6 viable lymph node cells. The suspensions were kept in the cold up to the moment that slides were prepared and incubated at 37°C. For the experiments with rabbits 6-73 and 6-74, 5 ml of cell suspension with 10^7 viable cells per ml were incubated for 2½ or 3 hours respectively with inhibitors in liquid medium. The cells were then centrifuged off, resuspended in BAF plus NRS, and samples prepared with indicator systems containing the same inhibitors. The procedures followed are those described above and in the following section on survival of cells *in vitro*.

The results are summarized in Table I.

Dinitrophenol was highly inhibitory at 10^{-3} M even if the cells were not preincubated in its presence. Under these conditions beta thienylalanine and

5-fluorouracil at 10^{-4} M were both considerably less active. When cells were preincubated with dinitrophenol in liquid culture for 3 hours before mixing with the indicator system, there was a significant decrease in viability judged by trypan blue staining, but the decrease in antibody formation was much greater than the decrease in viability. With the same conditions, puromycin at 2×10^{-4} M and actinomycin, 10 μg/ml, had relatively little effect on cell viability but were highly inhibitory to antibody formation. The inhibitors in

TABLE I

Effects of Inhibitors on Cell Viability and Antibody Production

Rabbit	Inhibitor*	Viable cells after pre-incubation‡	Active cells per 10^6 viable§	Activity relative to control‖
		per cent		*per cent*
5-31	None		148	—
	Ethanol, 1 per cent		180	—
	2,4-dinitrophenol, 10^{-3} M		8	4.9
	5-fluorouracil, 10^{-4} M		117	71
	β-thienylalanine, 10^{-4} M		58	35
6-73	None	82	61	—
	Actinomycin D, 10 μg/ml	87	18	30
6-74	None	92	100	—
	2,4-dinitrophenol, 10^{-3} M	61	4	4
	Puromycine, 2×10^{-4} M	74	2.6	2.6
	Actinomycin D, 10 μg/ml	90	13	13

* Final concentrations in the incubation or indicator systems are given.

‡ For rabbit 5-31, there was no preincubation in fluid medium; for rabbit 6-73 preincubation was for 2½ hours, and for rabbit 6-74 it was for 3 hours.

§ Based on survey of a total of 1 to 2×10^6 viable cells for rabbit 5-31 and 3 to 16×10^6 for rabbits 6-73 and 6-74.

‖ In the first experiment (5–31) the number of active cells in the control was taken to be 164, the mean of the system to which 0.01 ml of water ("none") and that to which 0.01 ml of 95 per cent ethanol had been added. Since ethanol was not inhibitory, the other experiments were referred to the activity of cells in the normal indicator system.

the concentrations used had no visible effect on lysis by preformed antibodies when tested as shown in Fig. 1.

Survival of Antibody-Forming Cells in Vitro.—It would be desirable to have an estimate of the absolute recovery of *in vivo* antibody-forming activity in our cultures. In one experiment we observed that the proportion of active cells was twice as high in the first crop of cells harvested as in the second crop obtained after more extensive mincing of the tissue.

To observe the time of survival of antibody-forming cells and the duration of antibody release *in vitro* under the conditions of our experiments, suspensions containing either ap-

proximately 3 or 6 × 10^7 cells per ml were prepared in medium 199 plus 30 per cent NRS. The rabbit (No. 5-30) from which these cells were obtained had been immunized and reinjected as described in Materials and Methods, but it had rested for 73 days following the first reinjection and was then given a second reinjection 4 days before autopsy. One ml samples of the cell suspension were mixed with 4 ml of Eagle's basal medium which had been adjusted to pH 7.4 with 5 per cent CO_2-air and were incubated in a CO_2-air atmosphere at 37°C in 50 ml Erlenmeyer flasks. After various times an 0.5 ml sample was removed for viable counts, and a suitable aliquot of the remaining cells was centrifuged, resuspended in 0.2 ml of BAF + NRS, and mixed with 0.8 ml of the indicator system for detection of antibody-forming cells.

The results of this experiment are summarized in Table II. The number of antibody-forming cells decreased faster than the number of viable cells, but

TABLE II

Survival of Antibody-Forming Cells in Culture

Time, hrs.*	0	½	1	4	22	22 (Cold)	46	72
Cells/ml (× 10^{-6}),	6.7	7.1	5.1	6.6	6.1	6.0	12	13
Viable, *per cent*	92	89	82	82	72	84	37	10
Active cells, /10^6 viable‡	1120	1060	720	780	470	700	120	<1
Activity relative to that at zero time§, *per cent*	100	92	57‖	62	33	57	4.4	<0.1

* Time from start of incubation at 37°C. The rabbit had been sacrificed about 2 hours before the start of incubation.

‡ Five samples containing a total of 1 to 2 million viable cells were counted at each time. A total of 1 to 2 thousand active cells was observed, and the range of the 5 samples was within ±20 per cent of the mean up through 22 hours.

§ $\frac{\text{Per cent viable}}{\text{Per cent viable at zero time}} \times \frac{\text{active cells}/10^6}{\text{active cells}/10^6 \text{ at zero time}} \times 100$

‖ In later experiments the survival was 76 and 85 per cent at 1 hour.

was still about 30 per cent of the initial value after 22 hours. By 46 hours, the viable cells had fallen to 37 per cent compared to 92 per cent initially, and the proportion of these viable cells still making antibody was only 10 per cent of that found initially. Storage in the cold prolonged both viability and antibody-forming ability.

Induction Phase in the Primary Response.—The following experiment was designed to observe the induction period and to relate this to the initial rise in serum antibody titer.

Six rabbits weighing 4 to 5 kg each were selected as having the lowest titers of natural anti-sheep hemolysin out of a group of twelve. When 0.6 ml of serum was incubated at 37°C for 30 minutes with 0.25 ml of 1:25 fresh guinea pig serum and 0.1 ml of 5 per cent sheep rbc in veronal buffer pH 7.2 in a total volume of 1 ml, lysis was complete with undiluted serum, partial at 1:5 and 1:10, and absent at 1:20 (or in one rabbit at 1:40). Of the twelve rabbits tested, 4 gave 100 per cent lysis at 1:10 to 1:80 compared to less than 1:5 for the six experimental animals. The selected rabbits were injected in the left hindfoot-pad with 0.2 ml of a

50 per cent suspension of washed sheep rbc and intravenously with 1 ml per kg of a 10 per cent suspension. One animal was sacrificed each day, and cells from the popliteal node and the spleen were examined for antibody formation. We had difficulty obtaining uniform dispersions of the spleen cells, and these preparations contained many large clumps of cells. Therefore, the quantitative reliability is not so good as for the lymph nodes. A sample of blood was obtained from the ear vein for serum titers every day up to autopsy.

The results are summarized in Table III. Within the limits of sensitivity of this assay there was no more than one antibody-forming cell for 3 to 4 million

TABLE III

Induction Period following Primary Injection

Day	1	2	3	4	5	6
Lymph Nodes						
Weight, *gm*	0.3	0.3	0.3	0.3	0.25	0.3
Total Cells extracted, ($\times 10^{-6}$)	33	38	100	32	89	68
Viable, *per cent*	82	78	73	77	76	70
Total viable in samples counted*, ($\times 10^{-6}$)	2.0	3.0	3.0	3.3	3.6	5.3
Total active counted/10^6 viable	0	0	0.33	7	81	31
Active cells in total extract‡	<16	<13	33	88	7200	2000
Total active cells in node§	<150	<100	100	800	20,000	9500
Spleen‖						
Total viable cells in samples counted, ($\times 10^{-6}$)	5.2	4.3	10.2	23	0.7	1.8
Total active counted/10^6 viable	0.19	0	0.49	0	105	154

* Viable cells/μl of assay system $\times$ 22 μl/sample $\times$ number of samples counted.

‡ Assumes that the present non-viable population contained *in vivo* the same proportion of active cells as observed for the present viable population.

§ Assumes 10^9 cells/gm of node.

‖ Spleens weighed 1.2 to 3.7 gm.

viable lymphoid cells up to 3 days after injection. An abrupt rise in activity started on the 4th day, and by 5 days there were 81 active cells per million in the lymph node and 105 per million in the spleen. The serum titers (Table IV) reflected the cellular activity with the first significant rise appearing on the 4th day after injection.

Various Observations on the Scope and Limitations of the Method.—Some experiments were done to explore the possibility of using this method to study the frequency of occurrence of individual cells making antibodies of two or more different specificities in response to various combinations of antigens under various conditions. Cells from lymph nodes of rabbits immunized against

pigeon erythrocytes by the same schedule as described for sheep erythrocytes lysed the former, but not the latter. However, the lysis of pigeon erythrocytes was much slower and less readily observed than that of sheep erythrocytes. Furthermore, the pigeon erythrocytes formed rather large clumps in the assay medium adding to the difficulty in interpreting the results when mixtures of pigeon and sheep cells were employed.

The lysis of sheep erythrocytes which had been coupled with sulfanilazo groups (SA-rbc) or with bovine globulin (BGG-rbc) was observed using preformed antiserum against SA-BGG on bits of filter paper as described in

TABLE IV

*Hemolytic Titers for the Primary Response**

Postinjection	Rabbits					
	5-51	5-56	5-59	5-60	5-61	5-54
days						
Preinjection	<5	<5	<2	<2	<2	<5
2	<2	2	4	4		
2½			2	2		
3		4	2			
3½	<4	2	2			
4	<4	8–16				
4½		16–18				
5	75–100					
5½	150–175					

* Values reported are the highest dilutions of serum giving 100 per cent lysis in the test described in the text. Several serums were lost including all those taken on the day of autopsy so the last titer is for the serum obtained the evening before the morning the rabbit was sacrificed.

Materials and Methods. Lysis of SA-rbc around papers with anti-SA–BGG was always partial although the area of lysis was several times as great as that around the papers with hemolytic serum against the sheep erythrocytes themselves. In tube tests the hemolytic titer of the anti-sheep cell serum against SA-rbc was 10- to 20-fold higher than that of the anti-SA serum. Limitation of the area of the reaction with anti-sheep rbc serum may result from its being more avidly absorbed to the red cell ghosts.

Lysis of BGG-rbc with the anti-SA–BGG serum was almost complete and was not visibly different from that seen with the anti-red cell serums. Moreover, the lysis of BGG-rbc by the anti-SA–BGG serum could be inhibited completely by the addition of 1 mg of BGG per ml of test system. We have done one experiment with SA-rbc using lymph node cells from a rabbit im-

munized with SA-human rbc stromata and two experiments with BGG-rbc using cells immunized with SA-BGG by schedules similar to that described in the methods for sheep rbc. None of these cell preparations gave lysis which could be shown to depend upon the groups coupled to the rbc. However, we do not attach much weight to these negative results, since we have occasionally failed to observe lysis of native sheep rbc by cells from rabbits immunized with sheep rbc.

When samples are taken at various times from a suspension of cells and analyzed for antibody-forming activity, it is not possible to know whether the active cells found have been releasing antibody constantly during all of the elapsed time. The proportion of active cells observed could be the result of a constant turnover. In order to observe immobilized cells in aerobic culture in the presence of the indicator system for prolonged periods, the following technique was used.

Rings of 29 mm outside diameter, 22.5 mm inside diameter, and 2.5 mm thickness with a groove around the outside edge were machined from stainless steel. A piece of dialyzing cellophane was tied over the ring by means of a nylon filament which rested in the groove. During this operation the ring was held on a brass rod about 150 mm long and 28 mm in diameter with a projection 2 mm high and 22 mm in diameter on one end. With the ring resting on a microscope slide moistened with culture medium, a sample of the indicator system containing immunized lymphoid cells was placed on the cellophane, covered with a round coverslip, and sealed with vaseline. The preparation was then put on a stainless steel support which held it about 3 mm off the bottom in a small Petri dish (Fig. 2), culture medium was added until it just contacted the under surface of the cellophane, and the dishes were incubated at 37°C in a humidified atmosphere of 5 per cent CO_2-air. Lysis could be observed *in situ* at low magnification, or for more critical observation the preparation could be examined under high power by removing it from the dish and placing it on a microscope slide which had been wetted with culture medium. It could then be removed from the slide and returned to the culture dish without disturbing the areas of hemolysis.

We have not yet detected any progressive increase in the number of active cells during periods up to 15 or 18 hours. However, we have so far made only a small number of observations by this technique. We anticipate that it may be useful when it is desired to add diffusible agents such as dyes, inhibitors, or fixatives to the cells *in situ* in the indicator system.

It has also been possible to make preparations for microscopic examination by spreading the indicator system on slides without coverslips, incubating in a humid atmosphere, and then fixing with osmic acid vapor.

To determine how long the cells will continue to release antibody in the assay system on slides, we have "erased" the plaques by moving the coverslip back and forth repeatedly over a distance of several millimeters and then observed reappearance of the lysis. When slides were erased after 30 minutes and then reincubated, the final number of plaques was almost the same as on

control slides. Slides erased after 2 hours had about 50 per cent of the original number of plaques following reincubation and even when erasure was delayed until 6 hours, a considerable number of plaques reappeared after 2 hours' further incubation. It is possible that the reappearance of plaques resulted from the action of deposits of lysin remaining in the gum. However, rather than large diffuse areas of lysis following reincubation, the plaques which reappeared were similar in size and definition to the original ones. Also, the observation that the number of active cells had decreased to 50 per cent of the original after 2 hours is consistent with the decrease in antibody-forming activity found when cells were observed in liquid cultures. It may be possible to resolve this question by continuous observation of the slides during reincubation or by the addition of inhibitors after erasure of the plaques in cultures incubated on cellophane as described above.

A total of 387 plaques on 11 slides were measured and classified as having clear, complete lysis or partial lysis. The observations were made after 18 hours in the incubator followed by 1 to 2 days at room temperature. Each sample was scanned across one diameter with the × 2.5 objective, and all plaques seen in the area so defined were evaluated. There was usually little difficulty in classifying a plaque as clear or cloudy. Twenty per cent of all the plaques were less than 0.11 mm in diameter; 50 per cent were between 0.11 and 0.20 mm; and 30 per cent were between 0.20 and 0.40 mm (disregarding two, which were over 0.40 mm). Most of the small plaques appeared cloudy as would be expected, since the diameter was less than the thickness of the preparation. But, 44 per cent of the largest and 60 per cent of the intermediate size were also cloudy.

Growth of the plaques was followed continuously in a warm stage at 37°C for one preparation, and it was observed that the increase in diameter was proportional to the square root of the time between about 10 minutes and 30 minutes. During this time, the diameter increased 3-fold from about 0.025 to 0.08 mm or in one case, from 0.04 to 0.12 mm. Lysis was first visible after 5 to 10 minutes and the curves extrapolated to zero diameter at between 2 and 7 minutes after the start of incubation. In another experiment, the edge of the lytic reaction along strips of paper impregnated with preformed anti-sheep rbc serum was followed similarly and the square root of time plots extrapolated to less than 1 minute.

Several antibody-producing cells were observed intermittantly for up to 4 hours after the start of incubation. Two of these were seen to undergo marked transformations after 2 to 3 hours. One of them (Fig. 3 *D*) appeared to be dividing to give a daughter of different morphology than the parent. However, these observations are of a very preliminary nature.

DISCUSSION

By means of the methods described here, it is technically feasible to survey the antibody-forming activity of many millions of cells from each of a number of experimental animals. The background was less than one plaque per 3 million cells from the popliteal nodes of rabbits selected for low natural anti-sheep hemolysin titers. This assumes that one discards atypical lytic reactions which occur in slides with inactive as well as with active complement. With the spleen, such atypical reactions were more frequent than with the lymph node. Furthermore, lysis developed around many of the clumps of cells which were present in our spleen preparations. This lysis occurred both with and without active complement and increased in extent for 24 to 48 hours. It seemed possible that the clumps of spleen cells might be providing both antibody and complement for the reaction. However, the slow rate and the duration of development of this lysis suggested it may have resulted from non-antibody lytic agents released from the splenic tissue.

The question arises whether the cells active in producing lysis in the assay system were synthesizing the lytic antibody *in situ* or simply releasing preformed or absorbed antibody. Lysis was sometimes visible within 5 to 10 minutes after the start of incubation. It has been observed that 20 to 40 minutes are required for labeled amino acids in the environment to be taken into cells and excreted as antibody (13). Therefore, this early lysis was probaby due to antibody which was "started" *in vivo*. On the other hand, the observation that the number of active cells was reduced in the presence of dinitrophenol suggests that an active energy-requiring process was involved in the release of the antibody. The reduction in the ratio of antibody-forming to viable cells with beta thienylalanine or puromycin indicates that incorporation of amino acids into protein was required (14). The inhibition by actinomycin suggests that maintenance of a supply of messenger RNA was required (15). Since these inhibitors did not interfere directly in the lytic reaction, it seems clear that the reactions we observed could not be accounted for by passive release of preformed antibody.

There has been considerable speculation regarding the processes occurring during the induction phase of antibody formation. Some recent observations suggest that the induction phase may be an artifact arising from the limitation in sensitivity of methods for titration of serum antibody. With ϕX bacteriophage in guinea pigs, the initial rise in antibody titer appeared to extrapolate to zero time (16). In the recent experiments of Jerne *et al.* (5) the increase with time in the numbers of cells forming hemolysins in mouse spleens also appeared to extrapolate to zero time. On the other hand, Taliaferro and Talmage (17) and Taliaferro and Taliaferro (13) found little or no incorporation of labeled amino acids into antibody protein during the 3 days of the induction period

in the secondary response of rabbits to bovine serum albumin. These observations were made in several species with a variety of antigens. However, if a true induction period in the general sense of a discontinuity in the initial stages of the process of antibody formation exists in one species with one antigen, it seems likely that, except for differences in degree, it would be a general phenomenon. In the present work, less than one antibody-forming cell was found per 3×10^6 lymphoid cells in the node through the first 3 days after injection, and on the 5th day there were 81 per 10^6. These observations are consistent with an absolute induction period during which there was no increase in the number of antibody-forming cells reflecting the delay in the appearance of detectable antibody in the serum. However, in view of the probable variation between animals, the present data are hardly sufficient to exclude a process which starts without lag with 1 cell at zero time and gives an increase of 6- to 8-fold per day for 5 days. If we assume that there are 10^9 cells per gm of node there would have been no more than 100 active cells in the whole node up to 3 days after injection. On the simplest assumptions this would require a generation time of 6 hours to give rise to 20,000 cells by 5 days, and would extrapolate to one active cell at 18 hours after injection. This generation time would be appreciably shorter than the minimum of 10 to 20 hours which has been found for mammalian cells in culture but may be possible for cells responding to antigenic stimulation (18). This would result in the appearance by 3 days of less than 0.5 per cent of the number of cells present by 5 days at the peak of the response, and would be consistent with the observations of both Uhr *et al.* (16) and Taliaferro *et al.* (13, 17). But, it seems unlikely that the response of the rabbit to sheep red cells could depend upon the almost unfailing presence of only such a very small number of competent cells. A process induced without lag in all 20,000 cells but which required 3 to 5 days to reach a detectable level would also be consistent with the data from this experiment. The alternatives discussed above are not entirely mutually exclusive. The problems of induction and the source of antibody-forming cells have been discussed recently by Nossal (2) and by Cochrane and Dixon (19).

At present we have no experimental evidence regarding what fraction of the total *in vivo* population of antibody-forming cells is recovered and detected by this method. The experiment on survival of antibody-forming cells in cultures indicates that 85 per cent of the active cells present at zero time survive ½ hour at 37°C; 60 per cent survive 4 hours, and the survival is better in the cold. On the other hand, a large fraction of the *in vivo* activity may be lost during preparation of the cell suspensions, and it seems possible that appreciable amounts of antibody might be produced *in vivo* by cells which made it at a rate too low to be detectable in the present assay system.

There was a considerable range in the lytic action between individual cells.

The smallest plaques seen after 18 hours' incubation out of 387 measured were 0.06 mm in diameter, and the largest were 0.4 mm (except for two larger which may possibly have resulted from the action of 2 cells). Assuming the rbc concentration to be uniformly 5 per cent by volume and taking the smallest hole to be a sphere and the largest to be a cylinder, the numbers of rbc lysed in the smallest and largest plaques would be respectively 100 and 15,000. The relationship between number of red cells lysed and the amount of antibody produced is probably complex and may depend in part on the local concentration obtained which would be a function of the rate of release. However, the range of activity observed must reflect a difference of many fold between the amounts of antibody produced by individual cells. It would be of interest to know whether this results from inherent differences between the cells or is due to cells being in different stages in the cycle of production.

Observations on the rate of growth of the lytic areas from individual cells suggested that there was no more than a few minutes' delay between the start of incubation and the start of release of antibody. This is consistent with the observation of Helmreich *et al.* (20) that cells start to release antibody without any lag when they are transferred from the cold to incubator temperature.

It was observed that about half of the antibody-forming cells gave lysis which was only partial, and this was true even of cells which gave the largest total volume of lysis. It seems possible that this could result from a very slow but prolonged release of antibody so that the total concentration never became high enough to give 100 per cent lysis. On the other hand, this may indicate that some cells produce antibodies which are specifically incapable of sensitizing a fraction of the sheep rbc.

This method of localized hemolysis should be applicable to studies of the formation of antibodies other than anti–red cell antibody and to the detection of the formation of several antibodies simultaneously. The experiments reported here on the scope of the method suggest that it will work with anti-BGG but that it will require further development for use with mixtures of red cells.

We anticipate that this technique could also be used with sensitized red cells and complement components to study the synthesis of complement.

SUMMARY

A technique is described which makes it possible to detect individual antibody-forming cells using a localized hemolysis reaction in a thickened culture medium containing sheep erythrocytes and guinea pig complement. This technique has the advantage over single cell isolation in that it is technically feasible to survey large populations in order to detect a very small active fraction. The cells can be observed continuously during the time of antibody release, and it appears that an estimate of the relative antibody-forming ac-

tivity can be made from the size of the areas of lysis. Experiments with metabolic inhibitors indicate that active synthesis is occurring rather than release of preformed antibody. Some experiments on the detection of antibody other than anti–red cell antibodies are reported.

This technique has been applied to a study of the induction period of the primary response of rabbits to sheep red blood cells. The results of this experiment are consistent with an induction period of 2 to 3 days during which there is no increase in the number of active cells in spleen and lymph node reflecting the lag in appearance of detectable serum antibody followed by an abrupt rise of 50- to 100-fold between the 3rd and 5th day. However, the present data are not sufficient to exclude various other mechanisms.

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Addendum.—We have now observed that inhibition of active cells in the presence of actinomycin D similar to that reported in Table I occurs at lower concentrations of the drug. Thus 0.5 or 1 μg/ml reduced the number of active cells in the mouse spleen or rabbit lymph node suspensions to 30 to 50 per cent of the controls after 4 hours preincubation. Although the numbers of active cells in controls also fell during preincubation (as in Table II), the acceleration in the rate of disappearance has now been reproduced in 6 independent experiments. Therefore, this appears to be a specific effect of the actinomycin on RNA synthesis (21) suggesting that the synthesis of antibody depends upon the continuous renewal of an RNA analogous to messenger RNA. Furthermore, at least part of this RNA may have a "half-life" as short as 4 hours.

We wish to acknowledge the assistance of Dr. Angenieta A. Biegel and Mrs. Grova M. Lewis with some of these experiments.

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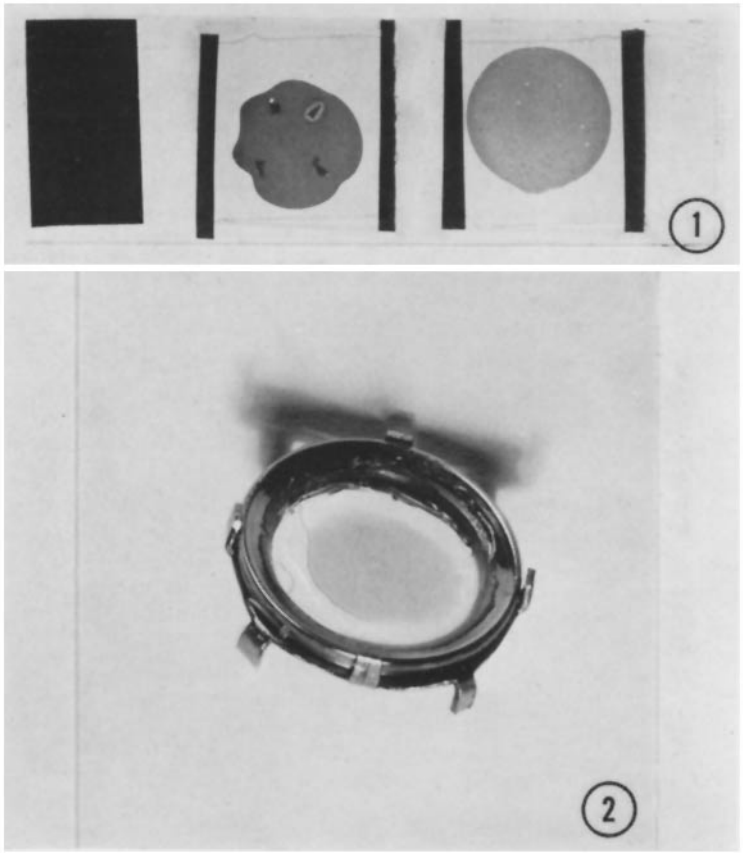
EXPLANATION OF PLATES

PLATE 74

FIG. 1. Two samples of antibody-forming cells in indicator system containing sheep red cells and complement mounted under sealed cover slips on a 25 x 75 mm slide as described in the text under Assay Procedure. The slide was viewed by transmitted light making the label and paper support strips appear black. The left sample is a control with 4 pieces of filter paper (black areas) containing various rabbit serums. The three triangles contain anti-sheep red cell serum (100 per cent hemolysin titer 1:3000). Starting with the piece at 1 o'clock and proceeding clockwise, the antiserum was respectively: undiluted, 1:5, and 1:25. The square at 11 o'clock contained NRS. Lysis is clearly evident only around the undiluted serum.

The sample on the right was prepared for counting plaques produced by the antibody-forming cells. The white circles are bubbles. With the aid of a hand lens, 28 light grey plaques of specific hemolysis could be counted on the original print from which this plate was prepared. The plaques were usually counted at a magnification of about 25 as seen in figure 3 *A*, but they were easily visible at a magnification of 2.5 to 3 as in Fig. 1 of reference 3. × 1.2.

FIG. 2. Apparatus for culture between coverslip and dialysing membrane as described in text under Various Observations on the Scope and Limitations of the Method. This picture shows the apparatus at slightly greater than actual size which was 29 mm outside diameter. × 1.3.



(Ingraham and Bussard: Specific detection of antibody-forming cells)

PLATE 75

FIG. 3 *A*. Area from the sample on the right in Fig. 1 viewed with a × 2.5 objective using semi-darkfield lighting as described in the text under Technique and Controls of the Method (total magnification approximately 25). With this lighting the plaques appear as dark areas at *1* to *5*, and *8* against a background of brightly lighted red cells. Small voids in the medium appear at *6* and *7*. The two clumps of red cells surrounded by voids which appear at *9* are the result of the absorption of small bubbles.

FIG. 3 *B*. Area from a thin preparation with mouse spleen cells made with *ca* 0.005 ml of indicator system and without paper strips to support the coverslip. There are bubbles at *1* and *4*, a void at *2*, and a specific plaque at *3* in the upper center. This was viewed with the × 2.5 objective as for Fig. 3 *A* after incubation for 1 hour. The background of red cells appears less brilliant and uniform here because the thickness is only about 0.02 mm compared to about 0.1 mm in 3 *A*.

FIG. 3 *C*. The specific plaque from Fig. 3 *B* viewed with a × 10 phase contrast objective (magnification approximately 100). Intact red cells are uniform, bright, refractile bodies appearing singly or in small clumps. As the red cells lyse they first fade in brilliance until they become quite black, and then gradually fade further until finally they almost disappear.

FIG. 3 *D*. Same area as in Fig. 3 *B* with × 40 phase contrast objective (total magnification approximately 400). Red cells can be seen in various stages of lysis from distinct black to faint grey circles. The large lymphoid cell in the center may have been dividing. Shortly before the picture was taken it resembled, except for size, the smaller cell toward 10 o'clock.

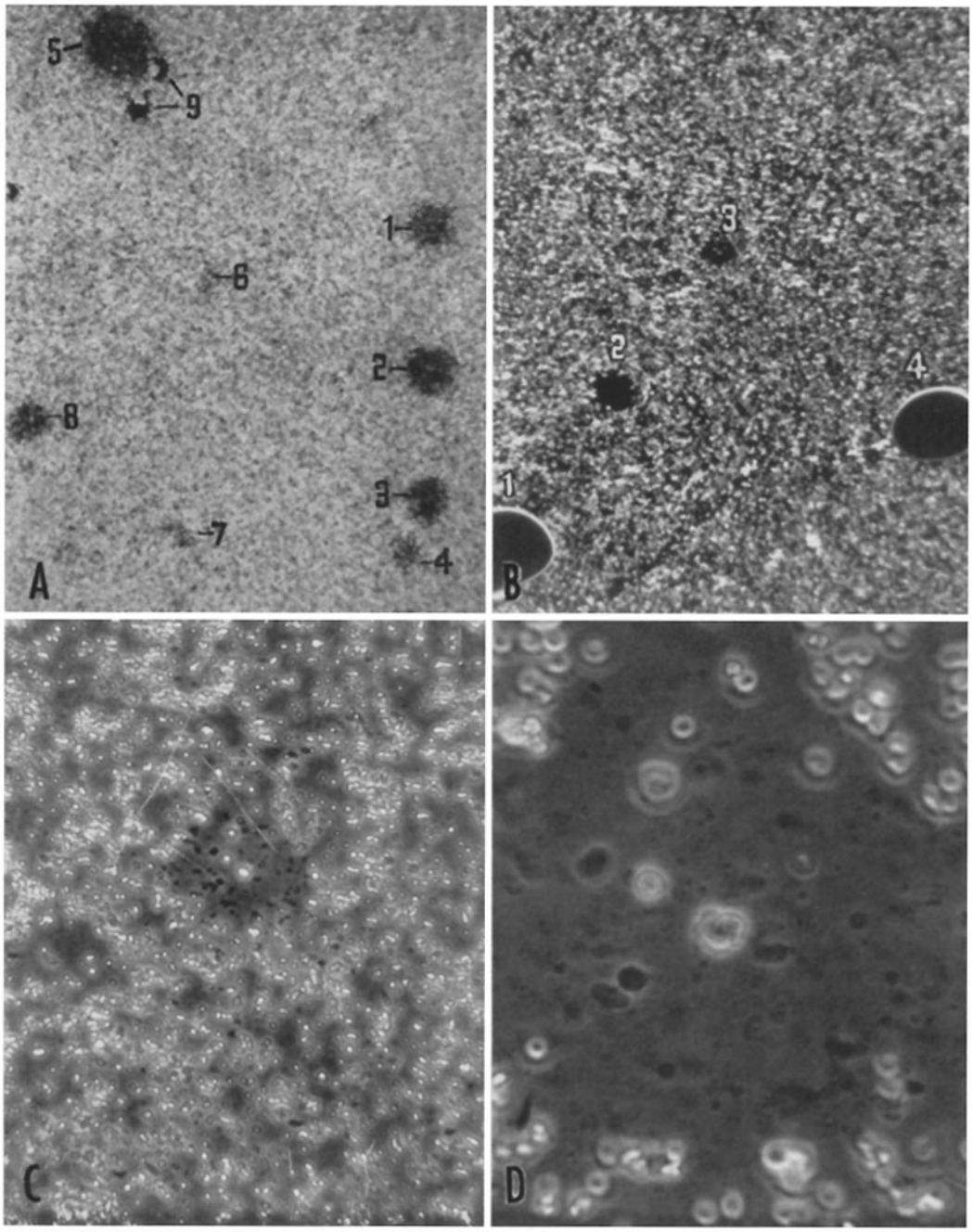


FIG. 3

(Ingraham and Bussard: Specific detection of antibody-forming cells)