Cytolysis in Agar of Thymus Cells by Antibody-Forming Cells[1] (34435)

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Hemolytic reactions exerted by antibodies and complement represent an essential part of agar plaque assay (1) by which lymphoid cells releasing antibodies against heterologous red blood cells can be recognized as plaques in agar. Increasing interests in the cell surface antigens of nucleated cells have led us to examine the possibility of applying plaque technique to such cells.

We report here a modification of the plaque assay by which distinct cytolytic plaques were obtained by using heterologous thymus cells as target cells. Results of parallel experiments in which ^{51}Cr-labeled thymus cells were employed in the autoradiographic plaque assay are also described.

Materials and Methods. In most experiments, mouse antirat thymus cell system was used. Cell suspensions of thymus and spleen were prepared in cold medium 199 by pressing tissues through stainless steel wire screen, 50 mesh, and then filtering through 200 mesh screen. The resulting cell suspensions were centrifuged at 1000 rpm for 10 min at 4° and resuspended in an appropriate volume of the medium. Inbred Swiss-Webster mice were injected intravenously with about 5×10^7 normal Lewis rat thymus cells (RTC). Mice were killed and their spleens were removed at various intervals thereafter. Some mice were stimulated intravenously with about 4×10^8 sheep red cells (SRC).

Plaque assay was carried out using 0.7% Bacto-agar (Difco Laboratories, Detroit, Michigan) prepared by adding 1.4% agar in distilled water to an equal volume of two times concentrated medium 199. DEAE-dextran (Pharmacia, Uppsala, Sweden) was added at a final concentration of 0.5 mg/ml (2, 3). To 0.3 ml of the agar mixtures kept at constant temperature in a 45° water bath were added 0.05 ml of a 10% suspension of RTC (approximately 2×10^7 cells) and 0.05 ml of an appropriate concentration of immune mouse spleen cells. The mixtures were spread evenly on microscopic slides, the surface of which had previously been coated with 0.1% agar. Slides were kept on a slide holder with their surface covered with medium 199 and were incubated for 1 hr at 37° in an atmosphere of 10% CO_2 in air. The medium was then removed and replaced with fresh medium containing fresh rabbit serum (complement) at a concentration of 25%. The incubation was continued for 45 min. The slides were washed in phosphate buffered saline, pH 7.3 for 15 min, fan-dried at room temperature and fixed in 95% ethanol for 15 min. The slides were then examined for plaques in the uniformly turbid layer of unlysed target cells. Experiments with mouse anti-SRC system were carried out by a similar procedure using a 5% suspension of SRC and guinea pig serum at a concentration of 10% as a source of complement.

In parallel experiments, RTC were labeled with ^{51}Cr (4) and used in the assay combined with autoradiography. The ^{51}Cr was supplied as sodium dichromate in isotonic solution by the Amersham Searle Company, Des Plaines, Illinois. The RTC were prepared as a 10% suspension in medium 199 to which 10% (v/v) fetal calf serum had been added. For labeling, 100 μCi of ^{51}Cr in a 0.1-ml volume were added to each milliliter of the cell suspension. The suspension was then incubated for 1 hr in a 37° water bath. During the incubation period the cells were

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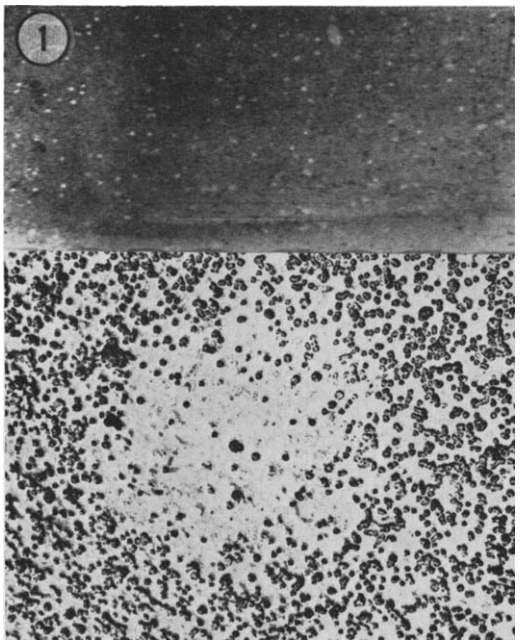

FIG. 1. Cytolytic plaques on agar slide in mouse anti-RTC system: Upper plate, 1.5× magnification. Lower plate, 240× magnification; single plaque with a central cell.

resuspended by gentle shaking every 15 min. After incubation the cells were washed once in 40 times their volume of medium 199 with added fetal calf serum and then resuspended to a 10% concentration. Small portions of the suspension before and after washing were removed for counting in a well-type scintillation counter to estimate the efficiency of the labeling procedure. The labeling of SRC was carried out in a similar manner except that phosphate buffered saline, pH 7.3, was used throughout, the cells were labeled as a 40% suspension, and incubation was for 30 min at room temperature with continuous shaking. Autoradiography was performed by placing the dried agar slides in contact with Kodak no-screen medical X-ray film NST-54 for 1 or 2 days. Following this exposure, the X-ray film was developed and fixed using Kodak X-ray solutions.

Results. Spleen cells of mice stimulated 4 days previously with RTC formed numerous plaques when they were mixed in agar with RTC and incubated in the presence of rabbit complement (Fig. 1, upper plate). The plaques were clear and circular; they had diameters ranging from 0.1 to 0.5 mm. Microscopic observation of individual plaques demonstrated severe destruction of target cells and a presence of a central cell in each plaque (Fig. 1, lower plate). Control slides without complement, with heat-inactivated complement or with RTC alone did not show plaques.

Studies with radiolabeled RTC were preceded by preliminary experiments with SRC system. In these experiments, hemolytic plaques could be also recognized by autoradiography (Fig. 2). In plaque assays which were carried out by use of ^{51}Cr-labeled RTC, discrete plaques were observed autoradiographically (Fig. 3). Those plaques which appeared on X-ray film represented areas where radioactivity of labeled target cells was lost. Plaques on an agar slide and those on an autoradiograph of the slide corresponded with each other in respect to the numbers and the sizes. Thus, the sensitivity of the two procedures in detecting antibody-forming cells appeared to be equal.

Mouse spleens removed at different times after stimulation showed increase in the number of plaques with time (Table I). As in the case of SRC, the peak number of cytolytic

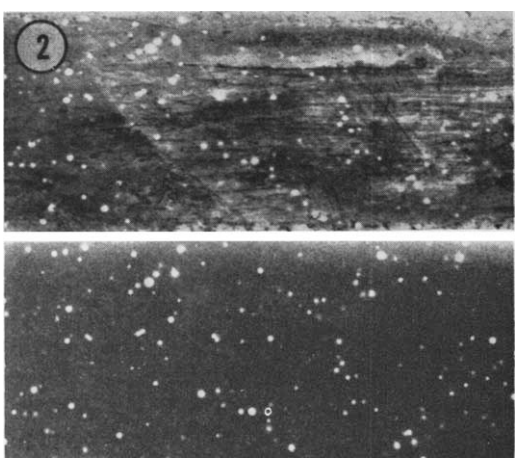

FIG. 2. Hemolytic plaques in mouse anti-SRC system: Upper plate, plaques on an agar slide. Lower plate, plaques on an autoradiograph of the agar slide.

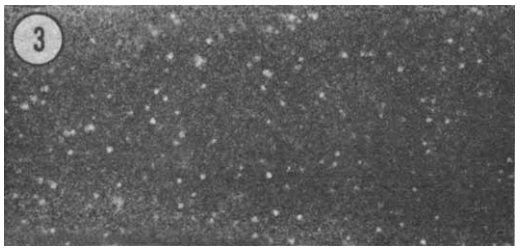

FIG. 3. Cytolytic plaques on autoradiograph in mouse anti-RTC system.

plaques was observed with spleen cells at the fourth day after stimulation.

We have observed that, in mouse anti-RTC system employed here, rabbit complement was more active in producing plaques than guinea pig complement. In fact, no plaques were obtained with guinea pig complement which was very active in producing plaques with sheep red cells. Similar observations were made in a mouse antirat red cell system (5).

Discussion. We have described two assay procedures which were satisfactory for detecting antibody-forming cells in mouse anti-RTC system. One procedure was to recognize localized cytolysis on agar slides by direct inspection. The other was to visualize such areas on autoradiograph by use of ^{51}Cr-labeled target cells. The sensitivity of two procedures was found to be similar. About 10^5 plaque-forming cells/total spleen was scored at the peak of primary response by these procedures. Direct visualization of cytolytic plaques may however depend on the numbers of mouse spleen cells incorporated into the agar because these cells will not undergo cytolysis. In preliminary studies not reported in "Results," we have investigated this possibility by adding increasing numbers of normal mouse spleen cells into the mixture of a fixed number of immune mouse spleen cells and rat thymus cells. It was found that at the ratio of mouse spleen cells to rat thymus cells of 1:5, plaques were still very clear. However, at the ratio of 2:5 or above, definition of plaques was reduced. Autoradiographic assay may have an advantage in such a case. Since the present assays make it possible to detect directly cytolysis of nucleated cells, further refinement of these techniques may provide a useful tool for the study of transplantation immunity, graft-versus-host reactions, and tumor immunity.

TABLE I. Numbers of Plaque-Forming Cells in the Spleens of Mice Stimulated with RTC.[a]

Days after stimulation	Mean nos. of cytolytic PFC/spleen	
	Scored on agar slide	Scored on autoradiograph
2	537	580
3	21,690	29,100
4	162,200	142,100
6	10,813	9700

[a] Mice were stimulated intravenously with 5×10^7 normal RTC. Three mice were used for the assay at each time point.

Summary. Rat thymus cells were used as target cells in Jerne agar plaque technique to detect and enumerate antibody-forming cells of mouse stimulated with this antigen. ^{51}Cr-labeled rat thymus cells were also used in these experiments to carry out plaque assay autoradiographically. The techniques demonstrated complement-dependent cytolytic plaques caused by antibodies released from immune mouse spleen cells and could be used to investigate kinetics of antibody-forming cells.

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