Enumeration of Cells Synthesizing Antiprotein Antibodies by a Modified Hemolytic Plaque Assay* (32744)

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The number of antibody producing cells in a lymphoid population can be quantitatively determined by a hemolytic plaque assay in a semisolid medium containing the target erythrocyte antigen. Zones of hemolysis (plaques) are produced: each representing antibody production by a single cell (1). The use of an antiglobulin reagent (2,3) has increased the sensitivity of the hemolytic plaque assay to include 7s IgG antibody as well as 19s IgM antierythrocyte antibody. Modifications have extended the use of this (3) antilipopolysaccharide assay to include measurement of antihapten (5), and antiglobulin antibodies (6) by coupling or coating of the erythrocytes with the proper antigens. To date, however, no generalized method which permits quantitation of the kinetics of early cellular antibody against a spectrum of protein antigens has been available. This report describes a simple technique which extends the scope of the hemolytic plaque assay to the study of the cellular antibody response to a wide range of protein antigens.

Materials and Methods. Indicator cells (sheep erythrocytes; SRBC) were prepared in isotonic pH 7.2 phosphate buffered saline (PBS) and conjugated with bovine gamma globulin (BGG:SRBC) or other antigen essentially by the method of Johnson *et al.* (7). Three ml of antigen of appropriate concentration (Table I) in PBS plus 0.1 ml of a 50% SRBC suspension were incubated for 1 hour at room temperature with 0.5 ml of 1-ethyl-3-(dimethylaminopropyl) carbodiimide hydrochloride (EDCI) (8). The cells were then centrifuged and washed two times and resuspended in 1% normal rabbit serum in PBS. The basic hemolytic plaque assay method was adapted from the method of Jerne and Nordin (1). The BGG:SRBC in 0.75% Difco agar were plated with immunized lymph node and spleen cells, incubated for 1 hour at 37°C; complement was added, and the plates were reincubated for 30 min. The plates were then left at room temperature for 2 hours and plaques were counted with the aid of a dissection microscope. Modifications of the original Jerne technique included the use of 2 mg of diethylaminoethyl dextran (4) in the plating medium; guinea pig complement diluted 1:10 in Tris buffer, pH 7.5, containing Ca^{++} and Mg^{++}; and the BGG coupled SRBC.

TABLE I. Concentrations of Antigens and Carbodiimide for Sensitization of Sheep Erythrocytes.

Antigen	Antigen conc. (mg/ml)	EDCI added in 0.5 ml of PBS (mg)
BGG	5	50
EGG	5	100
KLH	0.92	25
BSA	10	100

Immune cells were obtained from rabbits immunized with from 6–10 mg of alum-precipitated BGG, equine gamma globulin (EGG), keyhole limpet hemocyanin (KLH) or bovine serum albumin (BSA) distributed equally between the rear foot pads and intravenous route. Tissues were removed at various intervals after immunization, minced, filtered through gauze, counted, and adjusted to an appropriate cell concentration for optimal plating (5×10^7 to 5×10^5 cells/0.1–0.3 ml). Plaque assays on the cell suspensions of the pooled popliteal lymph node and spleen were performed in duplicate. Plaque counts were normalized to plaques per 5×10^6 cells for presentation. However, plates with from 30–300 plaques were routinely used for es-

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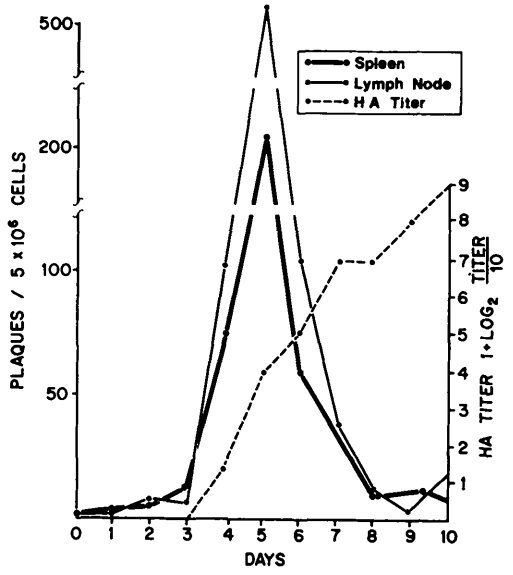

FIG. 1. Hemolytic plaque and serum antibody titers of rabbit anti-BGG serum and lymph node and spleen cell suspensions.

timation of total numbers. Serum hemagglutinin (7), and passive lysis (9) titers were determined with the same batches of cells used for plaque assays.

Results and Discussion. The cellular and serologic responses of 3 nonimmunized control and 14 experimental rabbits were studied after primary immunization with alum-precipitated BGG (Fig. 1). Protein reactive plaques characterized by central nucleated cells were first detected for both spleen and lymph node 3 days following immunization. In the spleen the 3-day response represented approximately a twofold increase over the average background whereas in the lymph node the response was greater than threefold. By day 4 the response was more obvious, representing a 40–50-fold increase over background. The validity of the plaque assay results was confirmed by the absence of lysis in controls with nonprotein-coupled SRBC, as well as in controls without complement and/or immune cells.

The generality of the method was indicated by data obtained with KLH, BSA, and EGG (Table II). The differences in plaque numbers may partially reflect differences in the sensitivity of the coupled erythrocytes. Experiments are currently underway to determine the point of optimal sensitization for these antigens, so that useful quantitative comparisons may be made at the cellular level. Preliminary data from experiments with mouse spleen cells substantiated the ability of SRBC covalently sensitized with BGG to form hemolytic plaques.

Recent investigations using immunologically-coupled antierythrocyte antibody as the indicator in a hemolytic plaque assay (6) failed to demonstrate maximal levels of cellular antibody until 8 days after antigenic stimulation. Assuming a minimal lag of from 24–72 hours between the maximal cellular response and maximal serum IgM response, this would suggest that the highest IgM levels would be present at days 10–11, postimmunization. However, available data (10–12) indicate that IgM antibody first appears and reaches a maximum level in the serum by 6–8 days after stimulation. The early cellular antibody response was indicated by the ECDI method to be greatest at day 5, correlating well with the levels of serum antibody. These results may perhaps reflect a greater sensitivity of erythrocytes covalently linked with antigen as opposed to those passively sensitized by antigen–antibody reaction.

Studies on circulating antibody indicate that the passive hemolytic assay described serves as a sensitive and accurate indicator of the early event of Ig synthesis in the intact animal. Although little or no antibody was detected in the serum at day 4 by hemagglutination or radioimmunoelectrophoresis (13), 5-day antisera had minimal hemagglutinin titers but appreciable levels of antibody activity as indicated by a modified antigen binding test (14). Considering the sensi-

TABLE II. Hemolytic Plaques Formed 5 Days after Immunization with Different Protein Antigens.

Antigen	Spleen	Lymph node	Spleen	Lymph node
	plaques/5 × 10^6 cells		plaques/organ × 10^{-3}	
BGG	290	945	48.1	113.4
EGG	54	64	4.0	8.0
BSA	147	36	11.3	3.7
KLH	39	117	5.6	16.8

tivity of the antigen binding tests for IgG, these results, substantiated by indications of slowly sedimenting antibody activity in spleen and lymph node lysate from 5-day BGG stimulated animals, are indicative of the early synthesis of low molecular weight antibody. These and other recent data (15–17) suggest that a clear-cut sequential synthesis of 19s IgM and 7s IgG is not a consistent feature of the early antibody response.

Summary. Cellular production of antibody to several proteins was studied using antigen-coupled erythrocytes in a modified passive hemolytic plaque assay. The kinetics of plaque formation correlated well with serum 19s IgM antibody, although a modified antigen binding test indicated early concomitant synthesis of 7s IgG antibody. Results of plaque assays for several protein antigens are presented.

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Occurrence of Cystathionine in Breast Muscle of Genetically Dystrophic Chicks* (32745)

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During a study of the free amino acids in breast muscle of chicks with hereditary muscular dystrophy (1), it was observed that an unknown ninhydrin positive constituent was present in dystrophic pectoralis muscle at 2 weeks of age but absent in normal pectoralis muscle. This substance declined sharply by 4 weeks of age and was no longer present at 6 weeks of age. In a further (2) study it was observed that the unknown compound was present in small amounts in both normal and dystrophic embryonic chick leg and breast muscle. It began to increase by the eighteenth day of incubation and had reached high levels just after hatching. Thereafter it declined in leg muscle from normal and dystrophic chicks and in normal breast muscle but remained high in dystrophic breast muscle so that by 2 weeks of age it had almost disappeared from all but dystrophic breast muscle.

The present paper describes the isolation and characterization of this substance.

Experimental

Solvent systems for paper chromatography. I. 95% ethanol:2 methoxy ethanol:15 *N* NH_4OH:H_2O (70:20:5:5 v/v) (1); II. *n*-butanol:glacial acetic acid:water (60:15:25 v/v) (3); III. methanol:2 ethoxyethanol:

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