Adaptation of the Hemolytic Plaque Technique for Enumeration of Immune Cells Responding to Heterologous Immunoglobulin Antigens. (32078)

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Knowledge of the cellular aspects of antibody formation has been greatly advanced by the development of hemolytic plaque or localized hemolysis-in-gel techniques. The original procedures allowed detection and enumeration of individual lymphoid cells producing antibody against sheep red blood cell (SRBC) antigens(1). Modification of the indicator red cells with certain bacterial somatic antigens permitted detection of immune cells responding to polysaccharide determinants(2). The further extension of plaquing techniques to chemically defined haptens provided additional information on the cellular response to other kinds of antigens(3), but response to the protein moiety of the immunogenic hapten-protein complex could be only indirectly inferred.

The development of anti-γ-globulin procedures(4) suggested that a similar system might be used to identify cells producing antibodies against an important category of protein antigens, the immunoglobulins. It was reasoned that immune cells producing antibody against heterologous immunoglobulin antigens would produce hemolytic plaques by passive hemolysis of erythrocytes which had been coated with these proteins. Using sheep red cells coated with sub-hemolytic amounts of mouse anti-SRBC serum and spleen cells from rabbits immunized with mouse immunoglobulins, it has been possible to develop such a specific detection system for cells producing antibody to these proteins and to study the cytodynamics of the immune response elicited by them.

Materials and methods. Adult New Zealand White rabbits were immunized with a single intravenous or intraperitoneal injection of 5 ml of a 50% suspension of autologous or homologous rabbit red blood cells coated with the mixed immunoglobulins adsorbed from 0.2 ml of hyperimmune mouse anti-rabbit RBC serum. The coated cells, bearing an estimated 0.1-0.5 mg of immunoglobulin, were washed 3 times with cold phosphate buffered 0.15 M NaCl to remove nonreactive serum proteins. Details of the procedure used to detect the appearance of plaque-forming cells have been described(3). An essential modification was made, however, by using indicator SRBC which had been coated with mouse immunoglobulins by exposing a 2% suspension of washed sheep erythrocytes for 1 hour at room temperature to an equal volume of mouse anti-SRBC serum diluted to a just subagglutinating (sub-hemolytic) concentration. Suspensions of cells in amounts which represented 1/25, 1/100, or 1/250 of the rabbit spleen, were plated in parallel on immunoglobulin-coated and uncoated SRBC. The serologic response was followed with passive hemagglutination and passive hemolysin titrations by using the same preparation of globulin-coated erythrocytes. Indicator cells which had been coated with subagglutinating amounts of human heterophile sera were used as a control of the serologic specificity of the system and the titers against these or normal SRBC were consistently zero. Furthermore, immunoelectrophoresis using whole mouse serum as antigen, revealed that these rabbit immune sera developed detectable precipitation arcs only against the mouse immunoglobulins.

Results. Data on the cellular and serologic responses evoked by primary immunization of 16 rabbits are summarized in Table I. Protein-reactive plaques with demonstrable central nucleated cells were first detected in the spleens of two rabbits 5 days following primary immunization. Although the magnitude and timing of cellular responses were variable, peak response was attained by the seventh or eighth day and detectable protein-reactive PFC activity had completely subsided by the 15th day. The numbers of background PFC

TABLE I. Cellular and Serologic Response of Rabbits Following Primary Immunization with Mouse Immunoglobulins.

Interval between immunization and test (days)	Natural background PFC/spleen*	Net protein reactive PFC/spleen*†	Immune cell index	Reciprocal of HA titer
0	2,625	—600	—.23	0
	2,200	—225	—.10	0
	650	—100	—.15	0
4	75	58	.77	0
	150	83	.54	0
5	450	225	.50	0
	1,350	—417	—.31	
	675	4,608	6.81	8
	1,475	14,325	9.70	8
6	550	260	.49	0
	125	83	.66	0
	2,300	—1483	—.65	0
7	75	892	11.9	4
	50	1,500	30.0	4
	125	13,208	106	32
8	1,150	36,000	31.9	512
	2,050	33,150	16.1	128
10	900	2,875	3.19	256
15	3,350	—517	—.15	0
	1,600	—200	—.13	0

* Each value represents the mean of 3 determinations.
† Corrected for background.

Protein-reactive PFC were developed on SRBC coated with subagglutinating amounts of mouse anti-SRBC serum. Hemagglutinin (HA) titrations were performed on identical globulin-coated SRBC. Background PFC were developed on uncoated SRBC. The immune cell index is the ratio of net protein-reactive PFC to spontaneous "background" PFC activity against SRBC.

per spleen directed against SRBC antigens were also variable. In order to assess more accurately the net increments of protein-reactive PFC over their natural "background" SRBC-reactive counterparts, an immune cell index has been calculated. This value is simply the ratio of the net protein-reactive response to the SRBC-reactive "background" and for control or non-responding animals it regularly assumes values either slightly above or below zero. This reflects an absence of discernible natural "background" PFC activity against mouse immunoglobulin antigens. Negative immune cell indices result from negative net protein reactive PFC values; both are attributable to limitations in the precision of counting. Eight of the 16 immunized rabbits provided unequivocal evidence for protein-reactive PFC responses; hemagglutinating activity against globulin-coated SRBC was demonstrable in each of these animals. Serum hemolysin activity was detected in only one of the 8-day animals and for this reason only passive hemagglutination titers have been reported. Passive hemolysin titers, however, might have provided information about antibody more closely analogous to that produced by plaque-forming cells.

Discussion. Previous studies of the immune response of rabbits to protein antigens (5,6,7,8) have provided information about the magnitude and timing of the serologic response and the molecular forms of the antibodies produced. Bauer and Stavitsky(5) and Shulman *et al*(6) found both 19 and 7S antibody to human serum albumin at 7 to 11 days following primary immunization. Mergenhagen *et al*(7), on the other hand, reported that primary response sera of rabbits immunized with human gamma globulin contained only mercapto-ethanol sensitive antibodies. Our findings are similar in that the hemagglutinating activity of these primary response sera was abolished by treatment with

0.1 M 2-mercaptoethanol at 37°C for 30 minutes while sera from rabbits which had received multiple immunizing injections retained full or partial hemagglutinating activity. It is of interest that the relatively small quantities of proteins used elicited an immune response without the use of adjuvant. Administration of the antigen in the form of globulin-coated cells may have provided an effective means for the activating of antibody producing cells(8).

The procedure employed for detection of cells producing antibody against immunoglobulin antigens initially constitutes an anti-globulin reaction. Performance of subsequent anti-globulin reactions(4), therefore, could not be assured to reveal additional PFC. Preliminary studies utilizing sheep anti-rabbit γ-globulin and spleen cells from hyperimmune rabbits have not provided, as yet, any clear evidence of such an additional population of cells. Consequently it remains to be determined whether the protein-reactive immune cells detected by this modified hemolytic plaque technique reflect the 19S, the 7S, or the total antibody-forming cell compartments.

A procedure is now available for study of PFC responding to an important category of protein antigens and the prospects are encouraging for the further development of plaquing techniques for additional classes of proteins. Thus, the presently available adaptations of the hemolytic-plaque technique now make possible a unified technological approach to the study of antibody formation against representative polysaccharides, haptens, and proteins at the level of antibody-forming cells.

Summary. Rabbit spleen cells producing antibodies against mouse immunoglobulin antigens were detected by a modification of the hemolytic plaque technique. The peak cellular and serologic response occurred 7 to 8 days following primary immunization and had subsided completely by the fifteenth day. The absence of spontaneous background distinguished protein reactive PFC from those reactive with erythrocyte antigens.

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