Lymphoid Cells Producing Antibody Against Simple Haptens: Detection and Enumeration

Abstract. *Enumeration of cells producing antibody against arsanilic acid was made feasible by the use of hapten-conjugated sheep erythrocytes for the localized hemolysis-in-gel technique. The time of appearance and numbers of these hapten-specific plaque-forming cells correlated closely with the development of arsanilic acid–specific serum antibodies.*

Localized hemolysis-in-gel (LHG) procedures (*1*), which make possible simple, practical, and highly sensitive evaluation of the dynamics of antibody-producing cells, are now a major technical resource for advancing the knowledge of cellular immunology.

Significant extensions of the original LHG technique have been accomplished by producing alterations in the erythrocytes used as indicator cells. Generally, this process has involved conferring on these indicator cells antigenic determinants additional to those native to the erythrocyte itself. The somatic polysaccharides of the Enterobacteriaceae have been shown to be effective in this regard (*2*). We now show that the LHG technique can also be applied to the enumeration of cells producing antibody against simple chemically defined haptens, for example, arsanilic acid.

Serum antibodies against arsanilic acid have been detected in vitro by hemagglutination (*3*) and hemolysis (*4*) of erythrocytes conjugated with arsanilic acid by diazotization. We now describe the use of such erythrocytes in the LHG technique.

Sheep erythrocytes were conjugated with arsanilic acid (Ars-SRBC) essentially by the method of Ingraham (*4*) except that protein (bovine serum albumin, BSA) was omitted from the buffer used for washing and storage of conjugated red cells. The conjugation reaction between hapten and erythrocytes was allowed to proceed for 15 minutes. The LHG technique was adapted from the method reported by Jerne and Nordin (*1*). The only modifications were incorporation of 2 mg of diethylaminoethyldextran into the immune-cell layer, use of guinea pig complement diluted 1 : 5 in tris-buffered 0.85 percent NaCl at *p*H 7.5, and the use of hapten-conjugated sheep erythrocytes.

The immune response to arsanilic acid was evaluated in adult New Zealand White rabbits immunized subcutaneously with bovine serum albumin conjugated with arsanilic acid (Ars-BSA). Rabbits were injected subcutaneously in the four dorsal quadrants with a total of 4 ml of a suspension of 20 mg of Ars-BSA incorporated into Freund's adjuvant containing 20 mg of killed, dried *Mycobacterium tuberculosis* cells.

The immune responses of these rabbits were assessed at various intervals after immunization; their serums, after absorption with sheep erythrocytes, were titrated for hemolysin and hemagglutinin activity against Ars-SRBC. Suspensions of rabbit spleen cells were plated in parallel on sheep erythrocytes and Ars-SRBC to ascertain the numbers of plaque-forming cells (PFC) developed. During the first 3 days after immunization no net differences were found between the numbers of PFC developed on Ars-SRBC and on the plain sheep cells, and no PFC against Ars-SRBC have been found in normal rabbits. The "natural background" PFC reaction against plain SRBC ordinarily ranged in number from 500 to 2000 per spleen in immunized rabbits.

Plaque-forming cells specifically reactive with Ars-SRBC were first detected on the 4th day after immunization, at which time the entire spleen contained an average of 8×10^3 specific PFC. The maximum number of PFC specific for arsanilic acid (arsanil-specific) in the spleens of rabbits injected with Ars-BSA was attained 5 or 6 days after immunization (Table 1). This immune response then subsided rapidly; by day 10, mean numbers of hapten-specific PFC were reduced to about one-tenth of the peak values.

Arsanil-specific serum antibodies, estimated by agglutination and lysis of Ars-SRBC, were first detected on the 5th day after immunization; their titers corresponded closely with rising numbers of PFC. After maximum levels were reached, these titers remained elevated throughout the 10-day period of observation. Of two rabbits immunized with 20 mg of soluble Ars-BSA without adjuvant, one developed 13×10^3 and the other 11×10^3 hapten-specific splenic PFC by 5 and 6 days, respectively. Their serums contained hapten-specific agglutinins at a dilution of 1 : 10, however, arsanil-specific lysins were below the level of detection. These results indicate that the use of adjuvant is not essential for the elicitation of hapten-specific cells detectable by the LHG technique.

Arsanil-specific PFC also appear in the spleens of rabbits immunized with arsanilic acid conjugated with hemocyanin, edestin, or human γ-globulin. Thus, the elicitation of arsanil-specific PFC is not dependent on the protein carrier. Low-efficiency arsanil-specific PFC (presumably due to immune cells that produce 7*S* globulin) have been observed after exposure of plates to an antiserum directed against rabbit γ-globulin (*5*). Such low-efficiency hapten-specific PFC have thus far been detected only after the 5th day of the primary response, but they frequently comprise the majority of all such cells detectable within the spleens of hyperimmune rabbits.

It is unlikely that arsanil-specific PFC are produced only in the spleen. Attention has thus far been focused on this organ primarily to facilitate correlation with titers of serum antibody. Possibly the cytodynamics of hapten-specific PFC will differ considerably in lymphoid organs other than the spleen, notably in lymph nodes after local administration of the antigen.

There are no a priori reasons for believing that our findings are restricted to arsanilic acid and to the rabbit. We have

Table 1. Cellular and serologic responses of rabbits immunized with 20 mg of Ars-BSA in complete Freund's adjuvant. Hapten-specific plaque-forming cells were developed on Ars-SRBC. The hemolysin and hemagglutinin (HA) titrations were performed on identical Ars-SRBC and represent the serologic response to the arsanilic hapten.

Arsanil-specific PFC per spleen	50% Hemolytic units/ml serum	Reciprocal of HA titers
2 Days after immunization		
0	< 10	< 10
3 Days after immunization		
0	< 10	< 10
0	< 10	< 10
4 Days after immunization		
9.5×10^3	< 10	< 10
7.8×10^3	< 10	< 10
5 Days after immunization		
2.4×10^3	50	40
2.5×10^4	79	20
5.2×10^4	186	80
8.5×10^4	56	80
1.4×10^5	740	160
3.2×10^5	141	80
6 Days after immunization		
3.8×10^4	790	160
7.5×10^4	830	160
1.4×10^5	57	40
2.4×10^5	455	160
8 Days after immunization		
1.6×10^4	415	160
4.2×10^4	275	80
10 Days after immunization		
2.7×10^3	465	160
2.4×10^4	370	80

evidence that rabbits immunized with protein-conjugated sulfanil and dinitrophenyl groups develop splenic PFC specific for these haptens (*6*).

Previous unsuccessful attempts to employ protein-conjugated erythrocytes in the LHG technique may now be subject to review. The conditions imposed by antigen coupling or conjugation have frequently so damaged erythrocytes that they become useless for the LHG procedure. Also certain steric and conformational factors are likely to affect the orientation and subsequent activation of complement on the red cell surface. Hapten-conjugated erythrocytes may be functionally suitable in the LHG technique, by virtue of the relatively small dimensions of the attached haptenic groupings and their consequent close proximity to receptors indigenous to the erythrocyte, which are themselves normally involved in immune hemolysis. By analogy, cells producing antibody to protein or synthetic polypeptide antigens might be readily detectable with the LHG technique by using indicator erythrocytes conjugated with appropriate peptides or with amino acid copolymers.

The efficacy of simple haptens in the LHG technique adds a new dimension to investigation of the cytodynamics of the immune response. Despite their simple structure haptens elicit a complex antibody response. Nonetheless they are representative of the best defined and most extensively studied model systems in immunology. The present work demonstrates that these model immune systems are now amenable to sensitive analysis in the study of antibody-producing cells.

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References and Notes

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Influenza Virus Purification with the Zonal Ultracentrifuge

Abstract. *Sufficient amounts of influenza virus (density, 1.185; size, 722S) can be highly purified (22,000 chicken cell agglutinating units per milligram of protein) with a zonal ultracentrifuge, used first in a rate process followed by isopycnic banding, to permit its detailed biological and physical-chemical evaluation.*

Harvey (*1*) first used the isopycnic-zonal centrifugation procedure to separate biological materials by banding and Brakke (*2, 3*) originated the rate-zonal centrifugation technique. Reviews of these procedures (*3*) indicate their importance. As performed in swinging-bucket rotors, these microfractionation techniques should be considered analytical methods rather than preparative procedures. They lead to purified fractions, measured as drops, which usually can be assessed only by methods which are highly sensitive to the relatively low concentrations of material present (*4*). Anderson and his collaborators have reported the continuing development of several new zonal-ultracentrifuge rotors (*5*). These new instruments are of general interest because samples of much larger volume now can be processed conveniently to obtain highly purified material for new or more complete evaluation.

We now describe our use of the commercially available B-IV rotor (*6*) (1.7-liter volume) for virus purification. Influenza virus, including types A (PR-8), A_1 (Ann Arbor), A_2 (Taiwan), A_2 (Japan 170), A_2 (Japan 305), B (Maryland), and B (Great Lakes), was grown in embryonated chicken eggs. Harvested allantoic fluid was first subjected to differential centrifugation in a Sharples Supercentrifuge with subsequent low-speed clarification of the resuspended virus pellet (*7*). This procedure for commercial vaccine production concentrates virus tenfold with respect to allantoic fluid and purifies it approximately 100-fold with respect to protein content. This partially purified product, usually containing 2000 chicken-cell agglutinating (CCA) units (*8*) per milligram of protein (*9*), was employed as starting material for the density gradient centrifugations.

In a typical experiment (Fig. 1) we

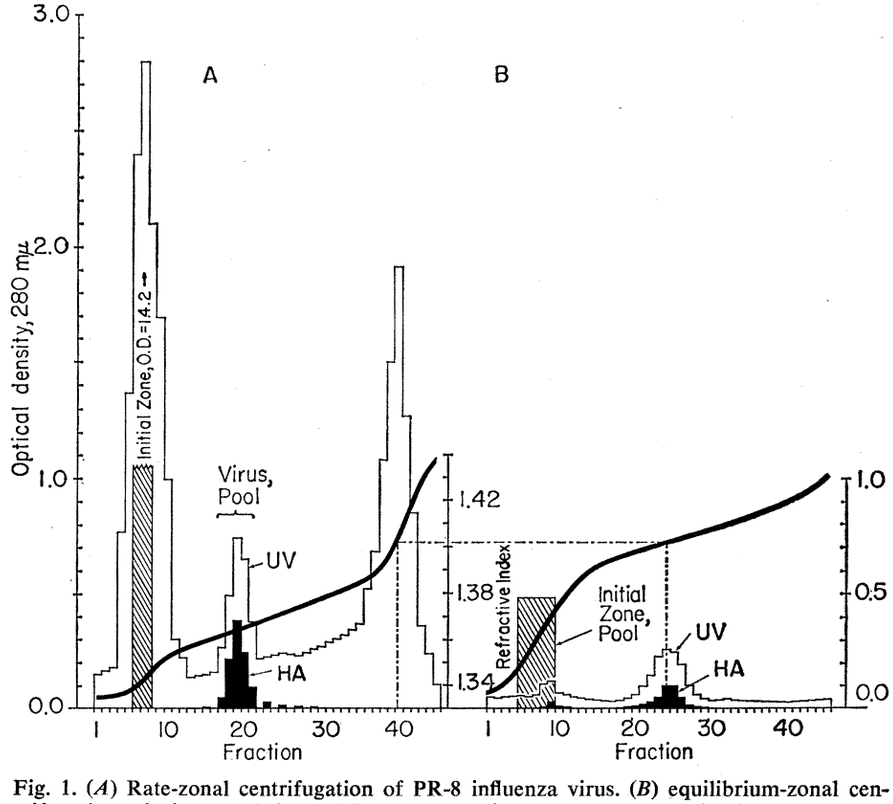

Fig. 1. (*A*) Rate-zonal centrifugation of PR-8 influenza virus. (*B*) equilibrium-zonal centrifugation of virus pool from (*A*). *UV*, ultraviolet; *HA* hemagglutination.