Detection and Localization of Antibody-Forming Cells to a Bacterial Endotoxin by an Immunobioautographic Procedure* (33620)

JERRY ALLEN, IRVING YOUNG, AND HERMAN FRIEDMAN
Departments of Microbiology and Pathology, Albert Einstein Medical Center, Philadelphia, Pennsylvania

Cellular aspects of the immune response can be readily examined by means of localized antibody plaque techniques in solid gel. Ingraham (1) and Jerne and Nordin (2) first described procedures whereby individual antibody-producing cells to sheep erythrocytes could be detected by localized hemolysis procedures using carboxymethyl cellulose gum or agar gel as a matrix. Recently, soluble antigens such as serum proteins, synthetic polypeptides, bacterial extracts, and chemical haptens have been utilized successfully for similar cellular studies of antibody formation (3–7). In such assays the target indicator system has consisted of carrier erythrocytes passively sensitized with the antigen used for immunization.

Viable microorganisms can also be used in a direct plaque procedure for detection of specific antibody-forming cells to gram-negative bacteria (8). Individual antibody-producing cells have been enumerated in spleen cell suspensions derived from mice immunized with *Escherichia coli* antigens, using living bacteria as the indicator in agar gel. The present report describes an immunobioautographic procedure which permits direct detection and localization of these antibody-forming cells to *E. coli* as specific foci or "clones" of bacteriolytic activity in intact spleen sections. Several characteristics of the immune response are described, including the effect of antigen dose and a comparison of the kinetics of appearance of antibody foci in spleens and that of individual plaque-forming cells and serum antibody titers.

Methods and Materials. Animals. NIH albino A mice, bred and raised as a closed colony by a local dealer, were used as the experimental animal. They were housed in groups of six in plastic mouse cages with wire mesh lids and fed commercial mouse pellets and water *ad libitum*.

Antigen. A lipopolysaccharide antigen derived from *E. coli* 0127:B8 was used for these studies. The endotoxin, prepared by trichloroacetic acid extraction procedures, was obtained from Difco Laboratories, Detroit, Michigan as a lyophilized powder. The preparation was maintained as a stock solution of 500 μg/ml in formalinized saline and diluted to the appropriate concentration immediately prior to injection.

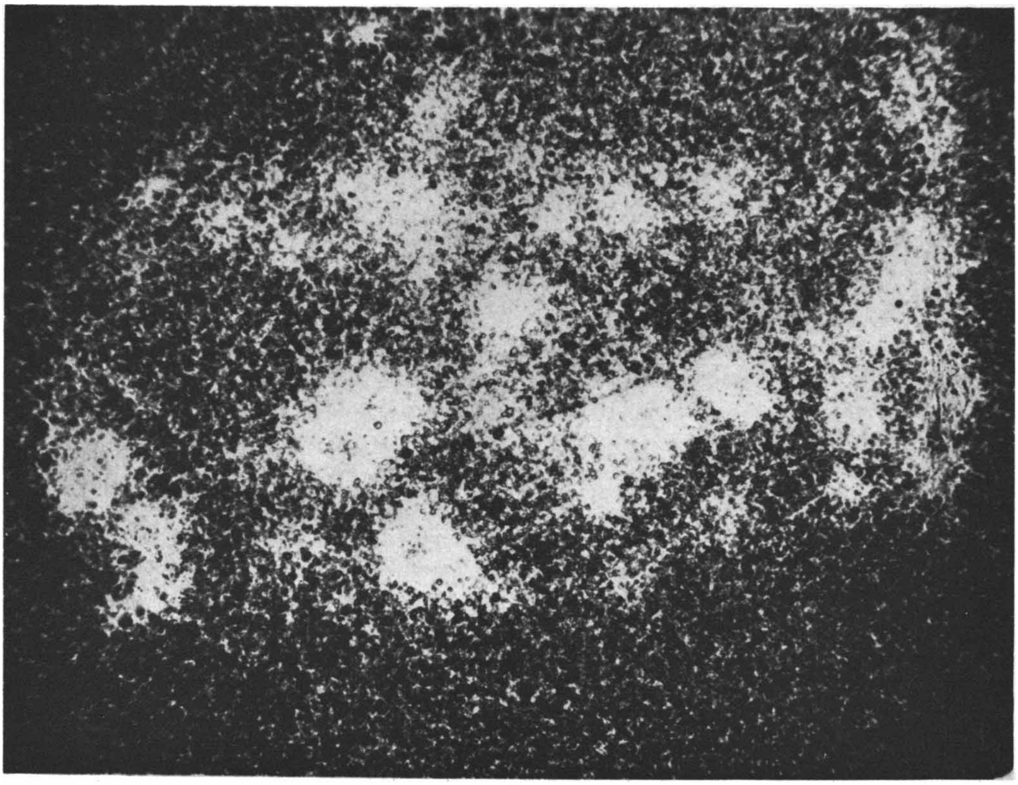
Immunization. The appropriate quantity of endotoxin in saline was injected intraperitoneally (i.p.) into groups of six or more mice at various times prior to sacrifice.

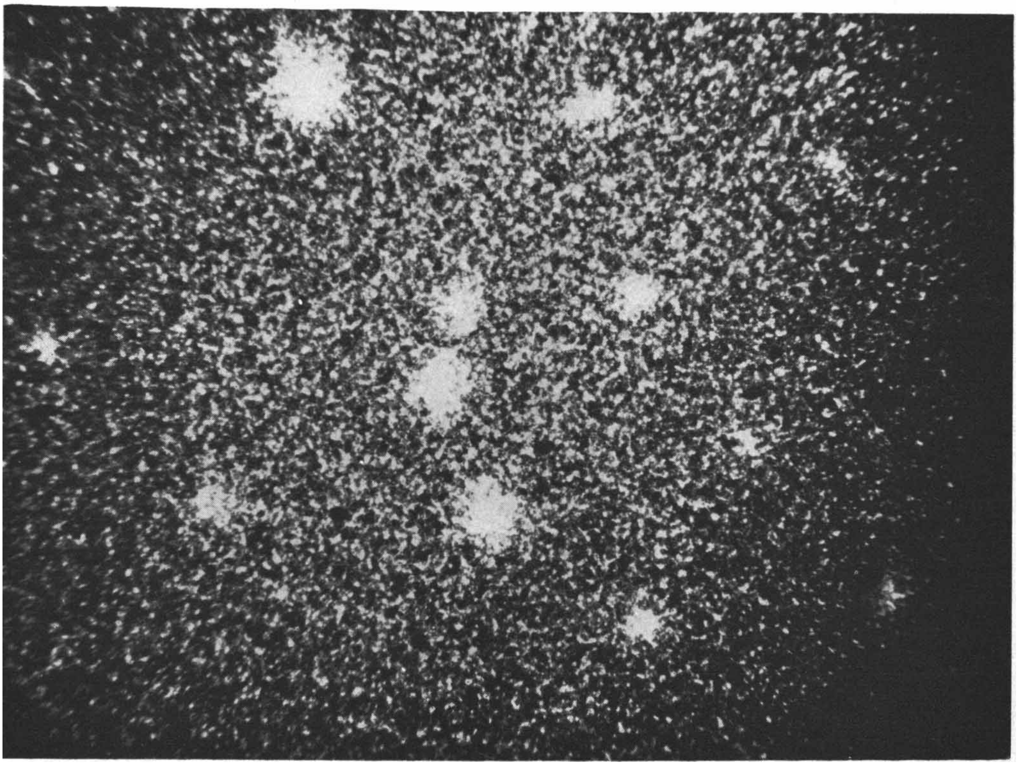
Serology. Blood specimens were obtained from individual animals by retroorbital venous puncture prior to immunization and at various time intervals thereafter. Serum samples were stored at —20° until used for titration. To determine agglutinin titers the serum specimens were serially diluted in duplicate, usually 1:2 to 1:4096, with 0.025 ml saline, using micro wire loops and microtiter plates. An equal quantity of an 0.1% suspension of a heat-killed culture of *E. coli* 0127:B8 was added to each dilution cup. The plates were incubated for 1 hr at 37° and overnight at 4°. The titers were recorded as the reciprocal of the highest dilution of serum which resulted in complete agglutination of the added bacteria.

Bacterial plaque procedure. For detection of antibody-forming cells to *E. coli* in intact spleen sections, mice were sacrificed at various time intervals after immunization. The spleens were obtained from individual ani-

---

* Supported in part by research grants from the National Science Foundation (GB 3278) and USPHS Fellowship No. 1-F2-AI-38012-01 to J.A.





mals at autopsy and cut into equal halves. One-half was quickly frozen at —60° with Dry Ice. Frozen sections, 6 μ thick, were cut in a cryostat and deposited onto the surface of a 100-mm diameter petri plate containing a base layer of solidified 1.4% brain heart infusion agar and an upper layer of 0.7% Difco Bacto agar. The upper layer consisted of 2.0 ml of agar containing 1.0 mg of DEAE-dextran (to neutralize the anticomplementary effects of the agar) and 0.1 ml of a washed suspension of an overnight slant culture of viable *E. coli* 0127:B8 (approximately 4×10^8 microorganisms). Following incubation for 1 hr at 37°, the plates were treated with a 1:15 dilution of guinea pig serum, as a source of complement, and further incubated for 1 hr at 4° and an additional hour at 37°. The complement was then decanted, the surface of the plates was washed twice with Hanks' solution, and the plates were incubated for 4–6 hr at 37° until a bacterial "lawn" developed. Areas of antibody activity to the *E. coli* appeared as clear foci of bacteriolysis in the indicator agar containing the microorganisms.

The number of individual PFCs to *E. coli* was determined by "teasing" the remaining half of each spleen in cold Hanks' solution, as described previously (4). The resulting single cell suspension was filtered through gauze into 15-ml conical centrifuge tubes and washed three times by centrifugation in the cold with Hanks' solution. Cell viability was estimated by trypan blue dye exclusion tests and the number of nucleated spleen cells per ml was determined with a hemocytometer. The volume of cells was adjusted so that the desired number for plating was contained in 0.1 ml of Hanks' solution. This was then added to 2.0 ml of warm melted 0.7% Bacto agar containing DEAE-dextran and viable *E. coli* (8). This mixture was carefully layered onto the surface of a 100-mm diameter petri plate containing the base layer of solidified brain heart infusion agar. The plates were then handled exactly as described above for detection of foci with spleen sections. Following incubation, single cells producing antibody against *E. coli* were detected and enumerated as zones of no growth in the bacterial "lawn." The number of antibody plaque forming cells (PFC) was calculated per million spleen leukocytes and per whole spleen.

Results. A typical "immunobioautograph" of spleen sections fom mice immunized with *E. coli* endotoxin revealed discrete foci of bacteriolytic activity (Fig. 1). Foci were easily distinguishable from the confluent colonies of bacteria covering the agar plates and those portions of the spleen where antibody-forming cells were absent. Most foci were generally about 0.5–1.5 mm in diameter. Microscopic examination revealed none or only occasional bacterial colonies in the areas of the antibody plaques.

The number of bacteriolytic foci per spleen increased rapidly as a function of time after immunization (Table I). Spleens of normal nonimmunized mice usually had a "background" of about 1–2 foci. There was a rapid increase in the number of such foci within a few days after immunization with 50 μg of endotoxin (Table I). Within 24–48 hr after immunization there were often 5–10 foci/spleen section. By the third day after immunization most spleens had a dozen or more foci. There were confluent areas of bacteriolysis, with few or no bacterial colonies over most of the spleen sections, by days 4–8 after immunization.

The antibody focus response was antigen dose dependent in that the fewest foci appeared when the lowest concentration of en-

FIG. 1. Appearance of antibody foci as discrete zones of bacteriolysis in agar plates containing mouse spleen sections and viable *E. coli* as the indicator: (upper) a spleen section of a mouse immunized three days before with 10.0 μg of endotoxin; (lower) spleen of mouse immunized with 1.0 μg of antigen. Six-μ sections were cut from the frozen mouse spleens and placed directly on agar plates containing the bacteria. Following treatment of the plates with complement and incubation at 37° bacterial colonies grew as a "confluent" lawn except in those areas where antibody-forming cells were present. Magnification ×35.

TABLE I. The Number of Antibody-Forming Cells and Specific Bacteriolytic Foci in Spleens of Adult Mice at Various Times after Immunization with Several Concentrations of *E. coli* 0127:B8 Lipopolysaccharide.

Antigen conc for immunization (μg)[a]	Antibody (foci/spleen section[b])						Antibody (plaque forming cells/10^7 spleen cells[b])					
	Day after immunization											
	1	2	3	4	6	10	1	2	3	4	6	10
50.0	3	5	18	C[c]	C	C	15	20	750	2108	497	185
10.0	5	8	9	C	C	17	24	36	759	1058	700	133
1.0	2	4	7	15	14	9	13	38	170	280	119	35
0.1	3	4	4	8	6	5	14	12	121	35	35	12

[a] Groups of 5 or more mice sacrificed on day indicated after i.p. immunization.

[b] Normal "background" of normal mice before immunization is 0–2 foci/spleen section and 10–25 PFC/10^7 spleen cells.

[c] Confluent bacteriolysis over spleen section (greater than 20–25 foci).

dotoxin was used for immunization (Table I). Animals receiving 10–50 μg of antigen had the largest number of foci per spleen during the first 2–3 days after immunization, and all had confluent areas of antibody activity by days 4–6. Animals injected with 0.1–1 μg of antigen had fewer foci and, in general, had only 8–10 discrete areas of bacteriolytic activity per spleen at the peak of the cellular antibody response (Table I). The number of foci per spleen decreased rapidly during the following few weeks, so that most spleens, even those from mice injected with 50 μg of endotoxin, had only a few foci.

There was a close correlation between the kinetics of foci appearance in spleen sections and appearance of individual PFCs as detected by the single cell assay. The peak number of individual PFCs was generally reached by the fifth day after immunization, regardless of the dose of antigen (Table I). However, the first significant increase in the number of individual PFCs usually did not occur until the third day after immunization, as contrasted to the more rapid rise in the number of antibody foci in the corresponding spleen halves. The total number of PFCs detected at the peak of the immune response was related to the amount of antigen used for immunization (Table I).

Specificity of the antibody response to *E. coli,* as determined both by the focus and PFC assays, was readily demonstrated by inhibition experiments (Table II). Incorporation of varying quantities of specific *E. coli* endotoxin into the agar plates prior to testing resulted in a marked suppression in appearance of both foci and PFCs. As shown in Table II, 50 μg *E. coli* endotoxin completely abolished appearance of foci in plates with

TABLE II. The Effect of Either Endotoxin or Antimouse Serum in the Test Agar on the Average Number of Antibody Foci Appearing with Spleen Sections from Mice Immunized 4 Days Previously with Several Concentrations of Antigen.

Dose of antigen used for immunization[a] (μg)	No addition	Endotoxin added to agar (μg)			Polyvalent antimouse γG serum (0.1 ml)
		50	10	1.0	
50	18	0	11	18	1
10	12	0	5	9	0
1.0	9	0	0	7	0

[a] Five mice immunized with each antigen concentration; sequential spleen sections tested, in duplicate, on agar plates with or without addition indicated.

spleen sections from mice immunized 4 days previously with either 1, 10, or 50 μg of antigen. Ten μg of endotoxin in the agar abolished appearance of foci with spleen sections from mice immunized with the lower concentration of antigen, as compared to sections from mice immunized with 50 μg of endotoxin. Incorporation of 1 μg of endotoxin into the test plates did not significantly decrease the number of foci, regardless of the dosage of antigen used for immunization. In similar experiments, incorporation of 0.1 ml of hyperimmune rabbit polyvalent antimouse gamma globulin serum into agar plates prior to incubation also abolished appearance of bacterial foci.

There was a significant increase in the titer of humoral antibody in sera of mice which had increased numbers of splenic antibody foci and PFCs. However, the rise in serum antibody was markedly slower than the cellular response. The earliest peak serum titer was usually reached by days 5–8 after immunization. There was usually no detectable increase in serum antibody until days 3–4 after immunization.

Discussion. The "immunobioautographic" technique described here permits direct demonstration of formation of antibody to a bacterial antigen in an immunocompetent organ such as the spleen. This procedure appears to be more sensitive than the single cell antibody method, or serum antibody titration. The sensitivity of the bacteriolytic focus technique with intact spleen sections is probably due to quantitative differences in the amount of specific antibody released by individual immunocompetent cells. It is probable that single cells forming antibody to *E. coli* may not synthesize sufficient bacteriolytic antibody during the first day or two after immunization to form a visible complement-dependent plaque. However, the accumulative effect of groups of such cells present at a single focus in a spleen section may permit detection of bacteriolytic activity. In additional studies, not reported here, use of thicker spleen sections, generally 300–600 μ thick, permitted even earlier detection of antibody foci during the first 24–48 hr after immunization. Sensitivity appeared to increase markedly as the thickness of the sections is increased. However, the resolution of zones of antibody activity decreased.

Histologic study of frozen sections was not feasible, as was done with foci to sheep erythrocytes (9), due to destruction of tissue in the agar brought about by action of bacterial growth. However, other studies using sheep erythrocytes sensitized with *E. coli* endotoxin indicated that most of the antibody foci appear to be related to the white pulp of the spleen, especially lymphoid follicles (10). Histologic studies with spleen sections sequential to those used on agar plates in this study also indicated that antibody secreting cells are present most often in sections of the spleen identifiable as lymphoid follicles and germinal centers.

It seems unlikely that antibody foci, as well as individual antibody plaques with dispersed cell suspensions, are caused by passive adsorption and then release of bacteriolytic antibody formed by different cells at distant sites. In control experiments, both with the *E. coli* and sheep erythrocyte systems, passive infusion of specific antibody into mice failed to sensitize cells so that they could be recognized as specific antibody-forming foci (Allen, Friedman, and Young, unpublished). In such experiments hyperimmune antiserum was injected into mice intravenously. One to 6 hr later the animals were killed and spleen sections were examined for antibody foci. There was no significant appearance of foci in such sections. On occasion it was noted that confluent bacteriolysis or hemolysis occurred with spleen sections late in the immune response. This appeared to be related to high titers of humoral antibody as well as to specific antibody-forming cells. In such instances sections of liver or kidney, which are ordinarily not considered organized antibody forming tissue, often had confluent antibody activity, indicating that serum antibody probably was responsible for at least part of this activity.

It is of interest to note the random but discrete distribution of antibody activity to *E. coli* in the spleens of the actively immunized animals. Such results suggest that there is a "clonal" proliferation of antibody-forming cells, presumably due to stimulation of individual antigen-sensitive precursor cells. Recent studies concerning distribution patterns of antibody-forming cells to sheep erythrocytes in spleens of actively immunized mice, or in X-irradiated recipients injected with cells from normal donors, revealed a similar random clonal distribution of hemolysin-forming cells (9–13). Further work in progress concerning transfer of antibody-forming cells or their progenitors may permit further understanding of the relationship between morphologic distribution of immunocompetent cells and biologic activity.

Summary. Specific antibody-forming cells to *E. coli* endotoxin were detected as discrete foci of bacteriolysis with intact spleen sections from mice immunized with varying concentrations of the antigen. Normal nonimmunized mice had an average of 1–2 antibody foci/spleen. Following immunization with 0.1–50 μg of endotoxin there was a rapid increase in the number of bacteriolytic foci, with a peak by the fourth to fifth day after antigen injection. Spleens of mice injected with 10–50 μg of antigen usually had confluent areas of bacteriolysis on the peak day. The animals receiving lower doses had fewer and more discrete antibody foci. Specificity was readily demonstrated by inhibition tests with specific antigen. In addition, the number of foci per spleen correlated with the increased number of single antibody plaque-forming cells, as detected by similar bacteriolytic antibody assays with dispersed cell suspensions. However, the increase in the number of single PFCs occurred later than did antibody foci. Similarly, the rise in serum antibacterial antibody, as detected by agglutination tests, occurred at a later time, with the peak usually between the fifth and eighth day after immunization. There was little or no increase in the level of serum antibody during the first 2–3 days after immunization.

The capable technical assistance of Mr. Jerry Rosenzweig and Miss Nancy Uszko during portions of this study is acknowledged.

---

1. Ingraham, J. S., Compt. Rend. **256**, 5005 (1963).
2. Jerne, N. K. and Nordin, A. A., Science **140**, 405 (1963).
3. Landy, M., Sanderson, R. P., and Jackson, A. L., J. Exptl. Med. **122**, 483 (1965).
4. Friedman, H., J. Bacteriol. **92**, 820 (1966).
5. Walsh, P., Maurer, P., and Egen, J. Immunol. **98**, 334 (1967).
6. Harrell, B. E. and Merchant, B., Intern. Arch. Allergy **32**, 211 (1967).
7. Golub, E. S., Michell, R. I., Weigle, W. O., and Dutton, R. W., J. Immunol. **100**, 133 (1968).
8. Schwartz, S. A. and Braun, W., Science **149**, 200 (1965).
9. Young, I. and Friedman, H., *in* "Germinal Centers in Immune Response," p. 102. Springer, New York (1967).
10. Friedman, H. and Young, I., Federation Proc. **25**, 370 (1966).
11. Nakano, M. and Braun, W., Science **151**, 338 (1966).
12. Clayman, H., Chaperin, A., and Triplett, F. R., J. Immunol. **97**, 828 (1966).
13. Berglund, S. E., Markey, P. A., and Mergenhangen, S. F., Proc. Soc. Exptl. Biol. Med. **126**, 84 (1967).

Received Sept. 4, 1968. P.S.E.B.M., 1969, Vol. 130.