deposition in those situations in which there is destruction of PMN leukocytes and a concurrent rise of soluble fibrin monomer complexes in the circulation.

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Comparison of Bacteriolysis, Passive Hemolysis, and Bacterial Adherence Colony Formation for Detecting Antibdy-Forming Cells* (33876)

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Considerable information is available concerning the nature of cells involved in antibody formation to antigens such as foreign erythrocytes, serum proteins and chemical haptens (1, 2). However, less consideration has been given to individual antibody forming cells to lipopolysaccharide antigens derived from bacteria. In this regard, most studies concerning immunity to microorganisms have dealt with serum antibody titers. From such studies it has been concluded that antibody to bacterial somatic antigens are mainly IgM, rather than IgG globulins (3–9). Even multiple injections of somatic antigens from gram-negative bacteria generally stimulate formation of IgM antibody. In contrast, serum proteins, haptens, and similar antigens appear to elicit mainly IgG antibody, often after a more or less transient macroglobulin response (1, 2).

Studies concerning cells involved in formation of antibody to various antigens have been facilitated in recent years by development of procedures which permit rapid detection of specific antibody plaque forming cells in semisolid medium such as soft agar or

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cellulose gum (10–12). Localized antibody procedures have been extensively used to study immunocytes secreting hemolysins to sheep erythrocytes. Indirect plaque methods with modified red blood cells as a carrier also have been developed and permit detection of antibody forming cells to serum proteins, synthetic peptides, chemical haptens, as well as bacterial somatic antigens (13–21). In this regard, Schwartz and Braun recently described a related but more direct antibody plaque procedure utilizing viable *Escherichia coli* as the target for antibody-forming cells secreting specific bacteriolysins (22). Diener has also described a direct plating procedure whereby lymphoid cells secreting antibody to flagellar antigens of Salmonella can be detected by a cytoadherence colony technique in agar gel (23).

Analysis of individual cells involved in systhesis of antibody to microorganisms should provide important information as to the cellular basis of immunity to infectious agents. The present study was concerned with evaluation of several of the new single cell procedures as rapid methods for detecting and enumerating lymphoid cells forming specific antibody to the somatic antigen of a microorganism. For this purpose groups of mice were immunized with the lipopolysaccharide antigen derived from *E. coli*. The number of splenic antibody forming cells was determined at various time intervals thereafter by(a) the direct bacteriolytic plaque technique,(b) the indirect passive hemolytic technique with lipopolysaccharide coated erythrocytes, and (c) the direct bacterial cytoadherence colony technique.

Methods and Materials. Animals. NIH albino A mice were used for these studies, essentially as described before (20). They were bred and maintained by a local dealer in the Philadelphia area. The animals were 3–5 months of age and generally weighted 20–25 g. They were housed in groups of six in plastic mouse cages with wire mesh lids and standard mouse bedding. The animals were fed commercial mouse pellets and water *ad libitum*.

Antigen. The test antigen was a lipopolysaccharide prepared by TCA extraction of *E. coli* 0127:B8 and was obtained from Difco Laboratories, Detroit, Michigan in a lyophilized form.

Immunization. Individual mice were injected intraperitoneally (i.p.) with 50 μg of a saline preparation of the lipopolysaccharide. This dose has been found in other studies to be optimum for immunization of these mice.

Serology. Blood was obtained by retroorbital venous puncture from each animal prior to and at various time intervals following immunization. Twofold serial dilutions of serum in saline (total volume 0.025 ml) were prepared with microwire loops and microtiter plates. To each cup was added 0.025 ml of a standard 0.05% suspension of a heat-killed overnight culture of *E. coli* 0127:B8. The plates were agitated to mix the bacteria and the serum dilution. The plates were then incubated for 2 hr at 37° and overnight in the cold. Titers were recorded as the reciprocal of the highest dilution of serum resulting in complete agglutination of the added bacteria.

An indirect passive hemolytic test also was performed, using sheep erythrocytes sensitized with specific lipopolysaccharide previously treated with 0.04 *N* NaOH for 18 hr. To each titration cup was added 0.025 ml of an 0.1% suspension of sensitized red blood cells. The plates were incubated for 3–18 hr at 4° and the titers were recorded as the reciprocal of the highest serum dilution resulting in complete agglutination of the sensitized red blood cells. Controls consisted of known positive and negative sera. Each serum was titrated in duplicate on at least 3 separate occassions. In addition, duplicate serum samples were treated for 1 hr at 37° with an equal quantity of 0.2 *M* 2-mercaptoethanol (2-ME) prior to either direct agglutination or indirect hemagglutination. The titer remaining after such 2-ME treatment was considered to be due to 7S IgG antibody.

Detection of antibody-forming cells. Three procedures were used for enumerating specific antibody-forming cells in spleens of immunized and normal mice. For all three procedures groups of 3–6 mice were sacrificed and individual spleens were obtained. Cell

suspensions were prepared by "teasing" the spleen into Hanks' balanced salt solution in the cold at 4° (20). The resulting single cell suspensions were clarified by filtration through sterile gauze into 15-ml conical centrifuge tubes. The cells were gently mixed with a Pasteur pipet and serially washed three times by centrifugation in the cold in Hanks' solution. After final centrifugation, the cells were added to an appropriate volume of Hanks' solution to prepare suspensions containing either 5×10^5, 5×10^6, or 5×10^7 nucleated cells/ml. The cells were then used for specific antibody plaque procedures, as follows:

Bacterial adherence colony (BAC) technique. To 1.0 ml of Hanks' solution containing a suspension of leukocytes was added 0.2 ml of an overnight culture of a washed suspension of *E. coli* (approximately 4×10^8 organisms). The mixture was incubated for 20 min at 4° so that antibody-producing cells, if present, would interact with the bacteria. One-tenth ml of the cell–bacteria suspension, presumably containing free bacteria, normal cells and immune cells with adhering bacteria, was added to 3.0 ml of warm, melted (42–48°) 1.4% Noble agar (Difco) prepared in brain–heart infusion (BHI) broth (Difco). The content of each tube was rapidly and carefully poured into 100 mm diameter plastic petri plates and incubated for 2–3 hr. The plates were then stained with 5–10 ml of an 0.025% solution of methylene blue stain in Merthiolate, diluted 1:1000. The plates were examined individually with a B & L Dynazoom stereo dissecting microscope, with 40× magnification and obliquely transmitted light. Under these conditions numerous microcolonies, about 8–10 μ in diameter, and presumably derived from single *E. coli*, could be readily identified in the agar layer (23). Larger colonies, about 20–40μ in diameter, were also seen in the plates containing spleen cells from normal and immunized mice. These were considered due to multiplication of the bacteria which had adhered to specific antibody forming cells (23). The bacterial adherence colonies (BAC) generally could be differentiated from single lymphoid cells or erythrocytes in the agar and from the smaller colonies presumably derived from the nonadhering bacteria. All BAC counts were made using an underlying grid on the microscope stage or by use of petri plates with an imprinted grid. The number of BAC per plate was determined and the total number per million leukocytes and per whole spleen calculated from the average of at least 3–4 plates.

Antibody plaque procedure. A bacteriolytic antibody plaque technique, essentially similar to that described initially by Schwartz and Braun (22), was performed for comparative purposes. In brief, 0.1-ml samples of the spleen cell suspensions were added to individual 10 × 75-mm tubes containing 2.0 ml of 0.7% warm, melted Noble agar and 0.1 ml of the overnight culture of *E. coli* 0127:B8 (approximately 4×10^8 organisms). This mixture was quickly poured into a previously prepared 100-mm diameter petri plate containing a base layer of 10–15 ml of solidified 1.4% BHI agar. The plates were incubated for 1 hr at 37° and then treated with 5 ml of a 1:15 dilution of guinea pig serum, as a source of complement. The plates were further incubated for 1 hr at 4° and 1 hr at 37°. The plates were then washed with cold sterile Hanks' solution and the plates were incubated at 37° for an additional 4–6 hr until a "lawn" of bacteria appeared on the surface of the agar. The discrete zones of bacteriolysis which appeared on the plates were considered to be due to specific bacterial antibody plaque-forming cells (B-PFC). The number of such B-PFC per plate was counted and used to calculate the number of PFC per million nucleated spleen cells and per whole spleen. Control plates consisted of agar with bacteria only. All spleen cell suspensions were tested in duplicate or triplicate at several cell concentrations.

Indirect passive hemolytic plaque procedure. For this technique, 0.1 ml of a test spleen cell suspension was added to 2.0 ml of warm, melted Noble agar (maintained at 42–48°) to which had been added 0.1 ml of a 10% suspension of target sheep erythrocytes, sensitized with *E. coli* lipopolysac-

TABLE I. Cytokinetics and Serum Antibody Titers of Mice Immunized with *E. coli* 0127:B8 Lipopolysaccharide as Determined by Several Procedures.

Time interval after immunization (days)	No. of mice tested[a]	Av no. of antibody-forming cells/spleen			Serum agglutinin titer (mean)	
		Bacterial cytoadherence colonies	Plaque formation		Bacterial agglutination	Sensitized RBC agglutination
			Bacteriolysis	Passive hemolysis		
0	12	2165	632	488	<1:4	<1:4
1	6	4890	689	585	<1:4	<1:4
2	6	11,763	3550	2650	1:4	1:4
3	8	38,500	12,430	9680	1:3	1:16
4	8	86,760	29,630	23,685	1:25	1:39
5	10	138,250	55,360	50,150	1:89	1:151
7	5	94,800	31,450	24,380	1:172	1:283
10	3	42,200	8340	1835	1:198	1:235
15	5	14,320	1552	1835	1:198	1:235

[a] Adult mice injected i.p. with 50 μg of antigen and sacrificed on day indicated.

charide antigen, and 1.0 mg of DEAE-dextran as an inhibitor of anticomplement activity agent. The red cells had been sensitized with an equal volume of NaOH-treated endotoxin (100 μg/ml) for 2 hr at 37°. The mixture of leukocytes and sensitized red cells was carefully poured into previously prepared 100-mm diameter petri plates containing a base layer of 15 ml of solidified Noble agar and DEAE-dextran. The plates were incubated at 37° for 1 hr, treated with 5 ml of a 1:15 dilution of guinea pig complement, and further incubated at 37° for an additional hour. Discrete zones of hemolysis appeared on plates containing spleens from immunized animals. The number of specific hemolytic plaque-forming cells (H-PFC) per 10^6 spleen cells and per spleen was calculated. Each cell suspension was tested in duplicate or triplicate at several cell concentrations. Control plates consisted of agar with sensitized red cells only and no spleen cells, as well as plates with unsensitized red cells.

Results. The number of antibody-forming cells detected by the three techniques with spleens from mice immunized with 50 μg of *E. coli* somatic antigen is indicated in Table I. There were at least 50,000 antibody-forming cells/spleen at the peak of the immune response in all mice tested, regardless of the technique used for enumerating these cells. However, the BAC method indicated the presence of at least twice as many antibody-forming cells as did either the direct bacteriolytic or the indirect passive hemolytic procedure.

Other related studies had indicated that the maximum number of antibody-forming cells to *E. coli* lipopolysaccharide usually occurred on the fifth day after immunization (24). In the present study, the number of antibody-forming cells detected by the three methods was studied in detail at close intervals during the first week after immunization. As shown in Table I, prior to immunization the number of antibody forming cells in spleens of normal mice varied widely. However, regardless of the individual animal tested, the BAC method consistently revealed 2–5 times as many "background" antibody-forming cells as did the other procedures. By 24 hours after immunization, spleens of most of the animals tested had a 2–3-fold increase in the number of BAC, indicating antibody-forming cells. This increase was not detected by the other two methods.

All three methods indicated a significant increase in the number of antibody-forming cells in all mouse spleens 48 hr after immunization. However, the number of BAC was considerably greater than the number of PFC (Table I). By 72–96 hr after immunization there was a 10–100-fold increase in the number of antibody forming cells in each spleen

tested, regardless of the method used. Nevertheless, the BAC procedure still indicated the largest number of antibody-forming cells.

Smaller groups of mice were sacrificed on days 7, 10, and 15 after immunization. The number of antibody-forming cells decreased markedly during this time period. However, the number of BAC was consistently greater than the number of PFC as the total number of antibody-forming cells diminished.

Individual serum antibody titers varied between 1:32 to 1:768 on days 5–7 after immunization, in all mice tested, with the mean titers approximately 1:90 to 1:280. There was no significant titer greater than 1:4 in sera of any mouse prior to the third day after immunization. There was no detectable serum titer prior to immunization. The level of serum antibody, both bacterial agglutinins and hemagglutinins, remained relatively constant from days 7 to 15 after immunization. All serum antibody titers were completely abolished by incubation with 2-ME for 30 min or longer.

Discussion. Localized antibody plaque procedures in agar gel or cellulose gum permits the rapid detection and enumeration of individual lymphoid cells secreting specific antibody to various antigens (10–12). The present study was concerned with comparison of several newer procedures with the potential of detecting specific antibody-forming cells to microbial antigens. Two of the procedures depended on direct interaction of individual antibody secreting cells with viable *E. coli* cells as a target. The third technique was an indirect procedure whereby the antigen used for immunization was coated onto sheep erythrocytes. Such modified red cells were then used as the target for complement mediated hemolysis in agar gel. In all three procedures the antibody secreting potential of individual lymphoid cells was determined by amplification procedures, *i.e.*, either lysis of bacteria, lysis of sensitized red cells, or formation of microcolonies in agar gel.

The study by Diener with a flagellar antigen derived from Salmonella had indicated that specific bacterial cytoadherence, followed by culture in agar gel, resulted in formation of bacterial colonies which could be differentiated microscopically from colonies which were formed from nonadhering microorganisms (23). It was reasonable to expect that similar results could be obtained with antibody-forming cells to somatic rather than flagellar antigens of gram-negative bacteria. The present experiments confirmed this expectation. Microcolonies developed around presumptive antibody secreting cells when spleen cell suspensions from mice immunized with *E. coli* lipopolysaccharide were incubated with viable *E. coli*. The initial incubation was for 20 min at 4°, during which time bacterial adherence occurred. These adhering bacteria, on individual lymphoid cells, were then detected by subsequent incubation in agar gel for several hours at 37°. The resulting bacterial colonies were detected microscopically and generally could be readily differentiated from the much smaller microcolonies derived from nonadhering bacteria.

The number of adherence colonies correlated well with the degree of immunization and the time at which the animal was sacrificed. In agreement with the interpretation of Diener (23), the results of these experiments suggest that appearance of microcolonies in the test agar plates is a sensitive indicator of individual antibody-forming cells. Although the original adherence colony technique described by Diener was presented as a method for enumerating antibody-forming cells to a purified protein antigen derived from bacterial flagella, it appears that the procedure is applicable to a variety of microbial antigens, including the lipopolysaccharide somatic antigens of nonflagellated gram-negative bacteria.

Comparative analysis was performed in this study with individual spleen cell suspensions to evaluate several antibody plaque techniques. Both the direct bacterial plaque procedure described initially by Schwartz and Braun (22) and the indirect bacterial plaque method with sensitized red cells were tested simultaneously with the BAC method. There was a good correlation between the number of BAC and the number of PFC as detected by the two plaque procedures. How-

ever, the number of BAC was consistently greater than the number of PFC, regardless of whether microorganisms or sensitized red cells were used as the target for complement mediated lysis.

Relatively large numbers of "background" antibody-forming cells were detected in the spleens of nonimmunized animals using all three procedures. Both the B-PFC and H-PFC method indicated several hundred plaque-forming cells in the spleens of normal animals, with a range from 220 to 680. The BAC assay with the same spleen cell suspensions indicated several thousand specific colony-forming cells.

It was not determined wehther a single cell which produced a BAC was also involved in the formation of a complement mediated antibody plaque with either bacteria or sensitized RBC. It is possible that different cell populations are involved in the different reactions. It is also possible that the same cells involved in BAC formation also are detected by the plaque procedures. However, the BAC procedure is undoubtedly much more sensitive and appeared to detect many antibody secreting cells which were not detected by complement dependent lytic reactions. This question is now being studied by replicate plating procedures.

It is of interest to note that the the serum antibody titers did not parallel the increase in specific antibody-forming cells until the third day after immunization. The single cell techniques showed a detectable increase 24–48 hr earlier. These results support the obvious conclusion that the cells detected by the BAC or PFC methods are the source of serum antibody. Similar relationships have been reached by many authors studying the cytokinetics of the immune response to sheep erythrocytes.

All of the antibody activity in the sera of the immunized mice sedimented as IgM globulins and was inactivated by treatment with 2-mercaptoethanol (Allen and Friedman, in preparation), similar to the observations of others concerning the macroglobulin nature of serum antibody to most gram-negative bacterial antigens. In addition, attempts to demonstrate 7S PFC by antiglobulin enhancement procedures using polyvalent anti-mouse immunoglobulin serum failed. These results suggest that all of the antibody-forming cells detected in the present study were probably producing IgM globulins. The specificity of the response was readily demonstrated, in other control experiments, not presented here, since increased numbers of either BAC or PFC did not appear when *Serratia marcescens* was used as the antigen.

Summary. Three procedures were compared in a study to determine the cytokinetics of antibody formation to the lipopolysaccharide antigen of *Escherichia coli* 0127:B8. A bacterial cytoadherence colony procedure, recently described for enumerating antibody-forming cells to Salmonella flagellin, was adapted to enumerate antibody-forming cells to *E. coli*. Antibody plaque procedures, using either viable *E. coli* or lipopolysaccharide-sensitized red cells as indicator targets, were also used. The bacterial cytoadherence procedure was more sensitive than the complement dependent plaque methods. Mice immunized with 50 μg of *E. coli* antigen had approximately 50,000 to 60,000 plaque-forming cells and over 130,000 colony-forming cells at the peak of the immune response 5 days after immunization. The first antibody forming cells were detected 24–48 hr after immunization. Normal mice had lower numbers of "background" antibody-forming cells. After a lag of 3 days serum antibody titers correlated with the appearance of antibody-forming cells.

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Vascular Volume, Blood Flow, and Resistance in Dog Kidneys During Constriction of the Renal Artery* (33877)

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It is generally accepted that renal blood flow remains relatively constant despite considerable changes in arterial pressure. Previous studies by Selkurt *et al.* (1) and by Shipley and Study (2) indicated that the constant blood flow is primarily due to changes in tone of the afferent arterioles while efferent arteriolar and postarteriolar resistance remain essentially unchanged. The observations by Thurau and Wober (3) were in accordance with these conclusions: they found a constant pressure in proximal convolutions and peritubular capillaries during autoregulation. The studies by Schmid *et al.* (4) indicated, however, that a change in postglomerular pressure contributed a little, although the results did not give support to the tissue pressure theory especially argued for by Hinshaw (5).

Recent investigations in man by Pedersen and Ladefoged (6) indicate that a greater part of the renal vascular bed other than the afferent arterioles changes its volume during regulation of blood flow in hypertension and renal artery stenosis. The aim of the present investigation was to measure to what extent renal vascular volume changes with resistance during constriction of the renal artery in acute experiments.

Material and Method. Three dogs, each weighing approximately 20 kg, were anesthetized by injecting 30 mg/kg of pentobarbital intravenously. Following endotracheal intubation, the left kidney was exposed by an oblique incision below the left curvature and the renal artery was isolated. A curved needle cannula was introduced into the artery peripheral to a tourniquet and pointed against the direction of the blood. The pressure in the renal artery was determined by connecting the cannula to a strain gauge manometer. The pressure in the aorta was measured by passing a catheter into the aorta via the femoral artery. After an injection through the cannula of 1–2 mCi of ^{133}Xe into the renal artery, renal blood flow was determined with the ^{133}Xe desaturation method described by Ladefoged (7). Mean circulation time for blood through the kidney was measured by use of ^{131}I-albumin as described by Pedersen

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