

Nonrandom Distribution of Vibriolytic Foci in the Spleen of Mice Lacking "Background" Antibody (35719)

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Previous studies from this laboratory (1, 2), as well as from others (3-5), have shown that lytic antibody to sheep red blood cells (SRBC) or to the lipopolysaccharide (LPS) somatic antigen of *Escherichia coli* appears in discrete foci in spleens of immunized mice. Such foci are detected in thin sections prepared from frozen spleens with a cryostat and placed directly on the surface of an indicator layer of agar containing the target antigen. Cytokinetic studies revealed that the number of antibody foci to SRBC or *E. coli* increases with time after immunization and that they are distributed randomly in the splenic sections. Furthermore, normal mice, prior to immunization, have readily detectable "background" foci to these antigens. There may be an average of 6/section (3). Nonimmunized animals also show significant numbers of individual antibody-forming cells in their spleen, as detected by single-cell plaque techniques (1-3).

A modified immunoplaque technique was developed recently for enumeration of individual antibody plaque-forming cells (PFC) producing bacteriolysins to *Vibrio cholerae* (6). No natural "background" of PFC to this organism was found in the spleen or lymph nodes of normal, nonimmunized mice. The present report describes a modification of this immunoplaque technique for detection of localized antibody foci to *V. cholerae* in frozen sections prepared from spleens of immunized mice. In accordance with the previous results obtained with single PFC, there were no antibody foci to vibrio detectable in the spleen of normal mice before immunization. After immunization, specific antibody foci developed and appeared to be distributed in a nonrandom manner in certain areas of the

spleen.

Methods and Materials. Animals. NIH albino A mice and inbred BALB/c mice, generally 5-8 weeks of age, were obtained from a local dealer or from Flow Laboratories, Rockville, Md., respectively.

Bacterial cultures. *Vibrio cholerae* strain Ogawa (J79) was provided by Dr. H. L. Smith of Thomas Jefferson Medical College, Philadelphia, Pa. The bacteria were maintained on carbohydrate-free medium (7, 8) at room temperature.

Antigen and immunization. A lyophilized whole bacterial cell vaccine was prepared. The cells were resuspended in sterile saline and concentrations were expressed on a dry weight basis. Mice were immunized intravenously (iv). A LPS antigen extract was prepared by ether extraction, according to the method of Ribic *et al.*, (9), and lyophilized. Saline suspensions of the LPS were used for inhibition experiments.

Antisera. Antiserum against the bacteria for passive antibody transfer experiments was prepared by immunization of mice with 1 mg of vaccine, either once or three times, at weekly intervals. Sera from six mice were collected 28 days after the single immunization or 12 days after the last of the three injections. The individual sera from each group were pooled and frozen until used. Antivibrio titers were determined, using the agglutination of viable bacteria as described by Goodner *et al.* (10), except that the test was carried out using the microtiter system rather than in glass tubes. Rabbit antimouse globulin serum was obtained from Microbiological Associates, Bethesda, Md. and normal rabbit serum from Difco Laboratories, Detroit, Mich. These sera were inactivated prior to

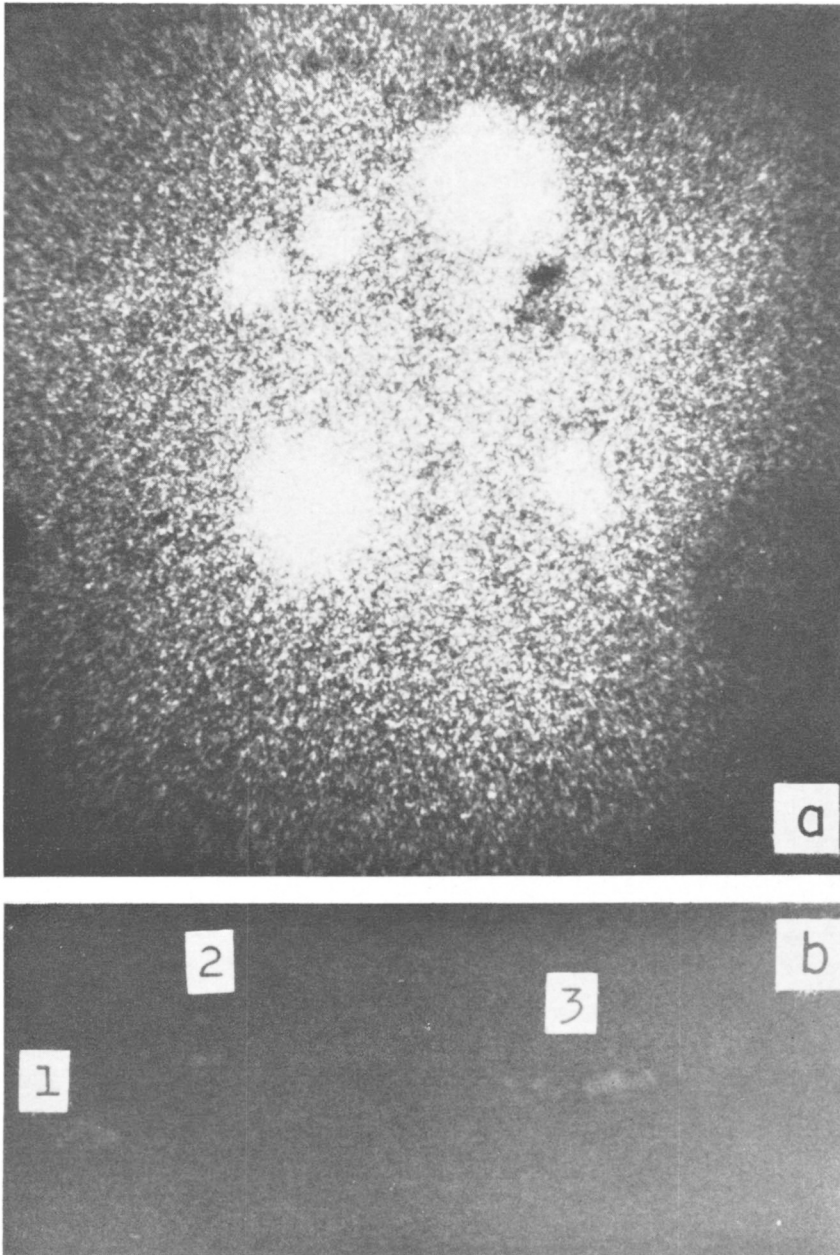


FIG. 1. Vibriolytic foci in one area of a spleen section on day 5 after immunization with 50 μg of cholera vaccine; 12.5 \times . (b) Longitudinal section of one spleen showing three areas of foci on day 4 after immunization with 5 μg of cholera vaccine. The areas (1-3) appeared in the same position in 20 sections taken randomly from the spleen. The average number of foci was 6 in area no. 1; 4 in area no. 2; and 15 in area no. 3.

use, for 30 min at 56°.

Vibriolytic plaque technique. The assay for individual vibriolytic PFC was performed exactly as described by McAlack *et al.* (6).

Antibody foci assay. Splens from normal or immunized mice were placed on 1 \times 1-cm pieces of Oncosponge (Histomed, Inc., Patterson, N.J.) in a position such that cut sec-

tions would represent the entire length of the organ. These were then frozen in place with a Freon spray (Alred Corp., Philadelphia, Pa.) and fixed in a cryostat (Cryo-Cut, American Optical Co.) at -18 to -20° . Sections ($6-7 \mu$ thick) were cut and transferred rapidly from the knife to a test agar plate. The plates were prepared previously by pouring 10 ml of brain heart infusion (BHI) broth containing 1.4% Bacto agar into 100-mm petri dishes (Falcon Plastic Co.) on a level surface and dried overnight at 37° . The indicator agar mixture, maintained at $47-49^{\circ}$, consisted of 2.0 ml of 0.7% Bacto agar, melted in Hanks' solution; 1.0 mg of DEAE-dextran; and 0.1 ml of a saline suspension of viable *V. cholerae* cells (approx 10^8 bacteria from 18-hr BHI cultures). One-tenth ml of bacterial LPS or rabbit antiserum was added in inhibition experiments. The agar-cell mixture was layered over the BHI base, allowed to solidify; and the plates then were inverted for 2 hr at room temperature before use. Plates with sections were incubated for 90 min at 37° and then treated with guinea pig serum diluted 1:15 in Hanks' solution. After 2 hr at 37° the complement was decanted from the plates, which were then incubated further at 37° until homogenous bacterial growth reached such an opacity that clear zones of bacteriolysis, located under the tissue sections, were sharp and clearly visible (4-5 hr). These foci appeared as lighter or darker areas in contrast to the bacterial "lawn" when observed under direct or indirect light, respectively.

Results. No antibody foci were detected in the spleens of 30 normal, nonimmunized mice, even when every spleen was explored by testing 20 to 30 consecutive sections through the entire organ. In mice injected

with 5-50 μ g of vaccine, the first foci appeared between 2 and 3 days after immunization as sharp zones of bacterial growth inhibition. The diameters varied from 0.1 to 1.0 mm (Fig. 1a). Incorporation of rabbit anti-mouse globulin serum into the assay agar suppressed approximately 50% of the foci detectable under the positive sections, whereas little if any effect was observed with the same amount of normal rabbit serum (Table I). Furthermore, over 50% of the foci were suppressed when 100 μ g of LPS from *V. cholerae* were incorporated into the agar (Table I).

Parallel groups of 6 to 10 mice were immunized with 5 μ g of vaccine, killed at different intervals after immunization, and assayed for the number of PFC per spleen and the number of foci per section, respectively (Fig. 2). There was a definite correlation of values between both assays. A similar relationship was observed in other experiments (unpublished observations) where the numbers of PFC and foci in two halves of individual spleens were determined.

The increase in the number of foci with time was not due to a local accumulation of humoral antibody in the tissue, as demonstrated by passive antibody transfer experiments (Table II). Normal mice injected 18 hr earlier with pooled antisera from immunized donors had detectable antibody titers in their serum but did not show any antibody foci or individual PFC in their spleen.

The first foci appeared in the splenic sections as isolated groups; this nonrandom distribution was maintained during the entire course of the response (Fig. 1b). Moreover, these groups of foci appeared in the same position in each sequential section from the spleen. Thus, by assaying 20 to 30 sections from each spleen it was possible to show the

TABLE I. Effect of Antiglobulin Serum (RaM) or *V. cholerae* LPS on the Number of Detectable Foci.

Incorporated into agar plate ^a :	None	LPS	RaM	NRS
Foci/section ^b	36	13	14	30

^a Each plate incubated with 0.1 ml of either lipopolysaccharide antigen (100 μ g), rabbit anti-mouse globulin serum (final dilution 1:25); or normal rabbit serum (1:25); before testing for antivibrio foci.

^b One positive spleen used; at least 6-10 sections were assayed with each reagent, as indicated, and average number of foci was determined.

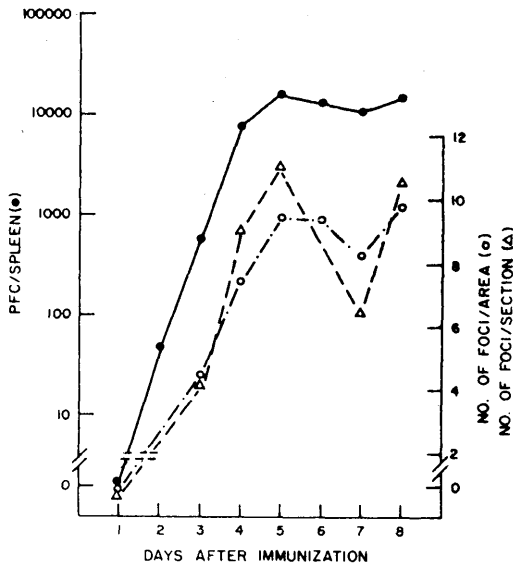


FIG. 2. Accumulation of PFC and antibody foci in the spleen after immunization of mice with 5 μ g of cholera vaccine: Assays performed in parallel with groups of 8 to 10 mice. Each point represents the average of one group. Foci were enumerated either per section (Δ); or per splenic area (\circ).

TABLE II. Effect of Passive Transfer of Anti-*V. cholerae* Serum on Immune Response.

Donor serum (titer) ^a	Av response in recipients ^b		
	Serum titer	PFC/spleen	Foci/section
Primary (1:8)	1:2	0	0
Secondary (1:1024)	1:32	0	0

^a Pool of sera from mice immunized once or repeatedly, as indicated in text.

^b Five mice per group bled and assayed 18 hr after iv injection of 0.5 or 0.1 ml of primary and secondary sera, respectively.

approximate three-dimensional distribution of individual areas of foci and to estimate both the number of such areas in the spleen and the average number of foci per area. There were an average of 2-3 areas/spleen in individual mice and this number remained unchanged during the first week of the response. In contrast, the average number of foci per area increased from 4 to 10 between days 3 and 5 of the response (Fig. 2).

Discussion. The complement-mediated immune lysis of the Ogawa strain of *V. cholerae*

appears to be due to interaction of specific antibody with either one or both of the two major cell wall antigens, designated A and B (11). Lack of "natural" or background serum antibody and antibody-forming cells (6), as well as an absence of background antibody foci, indicate that the immune reaction to these antigens may represent a true primary response. Thus this system appears to offer a valuable approach to the study of "virgin" antigen-reactive cells (ARC) capable of developing into antibody-producing cells.

Partial inhibition with soluble antigen and with antiglobulin serum incorporated into the assay agar indicates that the bacteriolytic foci are due to local production of specific antibody to *V. cholerae*. Furthermore, the definite quantitative relationship between PFC and foci shown in this model, as well as in previous studies with other antigens (2, 3), suggests that a focus may, in fact, be a cluster of accumulated antibody-forming cells.

It has been reported previously that the PFC response to the A and B antigens of vibrio can be enumerated independently in the same spleen by assaying one portion of a spleen cell suspension against the homologous Ogawa strain and the other portion against the Inaba strain possessing antigen A but not antigen B (6, 12). Application of this principle in a similar study for antibody foci to two strains of vibrio showed that each focus is immunologically monospecific, *i.e.*, each contains either anti-A or anti-B PFC, but not both (7). Thus, it is possible that the foci actually represent progeny of PFC arising from single ARC. Unfortunately, this type of assay for splenic foci cannot be used to estimate the maximum number of ARC in a spleen as accomplished by adoptive cell transfer techniques (13, 14). An increase in antigen dose results in an increase in the number of foci per section so that the number becomes uncountable over 30-40 foci/section. This is due to merging of individual foci. The same problem occurred in the assay using *E. coli* and sheep RBC as antigen (2, 3), except that confluent lysis and merging of foci generally occurred even with smaller antigen doses and earlier in the response.

The nonrandom distribution of foci in the spleen is consistent in this system and has not been described with other antigens. One explanation may be rather simple, *i.e.*, the antibody background to other antigens (several foci per section before immunization) may interfere with the distribution pattern of newly arising foci. However, Nakano and Braun observed that PFC against sheep RBC are accumulated nonrandomly in the spleen (15). There were 3–5 such "PFC-rich" splenic areas observed. This value corresponds well to our average number of 3 areas of foci/spleen. It seems, therefore, that single antibody-forming cells may arise in foci which are assembled in separate areas. There is no explanation presently available why specific precursor ARC are activated or assembled in a nonrandom fashion. However, this observation suggests that an unknown, but complex process of local maturation of cells, as well as mutual cell activation, may occur during the latent phase of the immune response.

Summary. Discrete foci of specific bacteriolytic activity are detectable with frozen sections from the spleen of mice immunized with *V. cholerae*. Development of foci can be suppressed substantially by incorporation of antiglobulin serum or soluble vibrio antigen into the assay agar. Passive injection of specific antibody into nonimmunized mice does not lead to foci formation. Furthermore, there are no background foci detectable in normal, nonimmunized mice. The number of foci increases with time after immunization and this increase correlates with the number of single antibody plaque-forming cells. The foci of antibody-forming cells are distributed in the spleen nonrandomly in 2 to 6 distinctive areas. The number of foci in each area increases with time after immunization. This suggests that antigen reactive precursor cells

may be activated only in a few limited areas of the spleen.

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