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Enumeration of Specific Antibody-Forming Cells of the Mouse Spleen after Stimulation with Protein Antigens[1] (39477)

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In the experiments to be reported here, cell counts trace the rise and fall of the population of antibody-forming cells in the red and white pulp of the spleen of the mouse during both initial and recall responses to the injection of antigen. Human IgG (HGG), heated β-galactosidase (BGz) from *E. coli*., and keyhole limpet hemocyanin (KLH) were used. In addition, the effects of a wide variety of doses on the distribution and number of cells making a specific response were studied for the cases of BGz and KLH.

Over the years White and his colleagues have made a systematic study of the localization of antigen and the appearance of antibody in the chicken spleen, using human serum albumin (HSA) as the antigen (1-3). The first antibody-producing cells occurred in the red pulp by the 30th hr, and only later did antibody appear in the germinal centers. They found traces of antibody, then increasing amounts of antigen, then more antibody in the germinal centers. Our data extend those of White and his colleagues by enumerating the cells and indicate the similarity of antibody-location in the mouse and the chicken. Urbain-Vansanten and her colleagues (4, 5) have counted the antibody-containing and immunoglobulin-containing cells in suspensions of rabbit spleen after antigen stimulation with several antigens; all were injected several times to achieve primary stimulation, without adjuvant. They found about five cells/thousand to contain antibody, and about 15/thousand to contain immunoglobulin not identifiable as specific antibody, but nevertheless in response to the antigenic stimulus in some perhaps indirect way. The work presented here confirms Urbain-Vansanten *et al.* and adds information about the location of the cells in the spleen.

Materials and methods. Antigens. Mouse γ-globulin was prepared from the serum of A.I. mice (Kyoto University Animal Center) by ammonium sulfate fractionation and DEAE cellulose chromatography (6). Purified β-galactosidase of *Escherichia coli* (360 units/mg βGz) was obtained from Worthington Biochemical Corp. (Freehold, New Jersey). The keyhole limpet hemocyanin (immunologically pure, KLH) was obtained from Calbiochem (Los Angeles, California). These were used as antigen without further purification.

Preparation of antisera and conjugates for immunofluorescence studies. Anti-BGz and anti-KLH sera were prepared in male albino rabbits and analyzed by double diffusion in agar. Neither cross-reacted with any antigen in mouse serum. Anti-MGG was tested against mouse serum after electrophoresis; it reacted with three antigens, all of which were in the region of γ-globulins and no others.

Globulin fractions of these antisera were conjugated with fluorescein isothiocyanate (7) as was HGG. Another fraction of the anti-BGz antibody was coupled with rhodamine isothiocyanate (8). These conjugates were fractionated on DEAE cellulose and concentrated by dialysis against polyethylene glycol (20,000 daltons).

Animals and immunization. Both sexes of inbred mice (body weight 18-25 g) were used in this study. Inbred C_3H mice were obtained from Kyoto University Animal Center (Kyoto) and Jackson Laboratories (Bar Harbor, Maine). A/Jax mice were obtained from Jackson Labs. Experimental animals in one series were given primary injections of 5 mg of soluble HGG into a tail vein. In other series heated BGz or KLH in

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various doses was administered intraperitoneally; for the secondary response to BGz, 0.1 mg heated BGz was given ip in complete Freund's adjuvant (Difco Labs) as a first dose, followed by another dose without adjuvant 4 weeks later.

Groups of two to three mice were killed on the days specified after secondary stimulation. Dose response curves for these two antigens were also carried out using two or three mice per dose.

Preparation of sections. Spleens were removed and cut lengthwise into three strips by a sharp razor. The middle strip of each specimen was placed quickly in cold 95% ethanol, held for 18 hr at 4°C, and embedded in paraffin according to the procedure of Sainte-Marie (9). Longitudinal sections (3–4 μm thick) were cut, deparaffinized with xylene, and hydrated through successive ethanol baths.

Staining with fluorescein-labeled conjugates and fluorescence microscopy. For the detection of anti-HGG antibody sections were exposed to labeled antigen, i.e., fluorescein-labeled HGG that had been absorbed twice with mouse liver or spleen powder. The exposure continued overnight in the cold with dilute conjugate (about 0.25 mg protein/ml). In the case of BGz-injected mice, sections were first exposed to rhodamin-labeled anti-BGz to detect any antigen remaining from the injection; none was found. They were then stained by the "sandwich" technique for anti-BGz (0.1 mg/ml BGz in PBS with 10% fetal calf serum for 2 hr, washed and then exposed to fluorescein-labeled anti-BGz for 1 hr).

For anti-KLH antibody, similar procedures were used without preliminary exposure to labeled anti-KLH. For MGG, sections were stained with fluorescein labeled anti-MGG for 2 hr or overnight. All incubation was carried out in a moist chamber at room temperature.

The stained slides were washed with PBS, mounted in glycerol containing 10% PBS, and examined under a Zeiss fluorescence microscope equipped with an HBO 200 high pressure mercury vapor lamp (Osram Company, Berlin, Germany) using a Corning 5840 exciter filter, a dark field condensor, and a Zeiss 41 barrier filter.

The specific stained cells were counted throughout the area of a section under a fluorescence microscope using a 12.5 × ocular and a 40 × objective. The approximate size of the section was estimated with the aid of a piece of graph paper ruled in millimeters. At least three good sections were examined per spleen.[5]

Results. The spleen consists of two main tissues, the white pulp, which sheathes the arteries and their small branches as they enter and ramify, and the red pulp, into which they empty blood. In addition, it is possible to identify a marginal zone, a broad ill-defined junctional tissue lying between the white and the red pulp. It receives much of the blood and is the initial area of concentration of antigen, and of the differentiation of lymphoid cells and macrophages. In the white pulp, round collections of lymphoid cells develop, the lymphoid follicles, and in their centers sometimes germinal centers. Around the germinal center is a cuff of small lymphocytes called the "mantle layer." (10).

The primary response. The first cells containing detectable antibody against HGG were found 8 hr after the injection of antigen in the red pulp; They were large mononuclear cells with a large nucleus and a thin rim of cytoplasm. Some of them showed fluorescence in only a portion of the cytoplasm. They occurred singly, their number slowly increasing during the first 4 days. Between the second and the fourth days the number of cells in the red pulp increased about twice, but now the gains began to shift to the white pulp; here during the same period the number increased 20 times. By the eighth day there were four times as many cells in the white pulp as in the red. This represented the peak; thereafter, the reaction gradually subsided (Fig. 1).

[5] For convenience, we have calculated roughly how many spleen cells the unit area of the spleen sections corresponds to. Assuming that the area of a spleen section is occupied by cells with a diameter averaging 10 μm, we estimate that 100 mm^2 of surface area corresponds to about $1–1.3 \times 10^6$ spleen cells. Abbreviations used in this paper: HGG, human gamma globulin; BGz β-galactosidase of *E. coli*; KLH, Keyhole limpet hemocyanin; MGG, mouse gamma globulin; DEAE, diethylaminoethyl cellulose; F, fluorescein; FITC, fluorescein isothiocyanate; PBS, phosphate buffered saline pH 7.2.

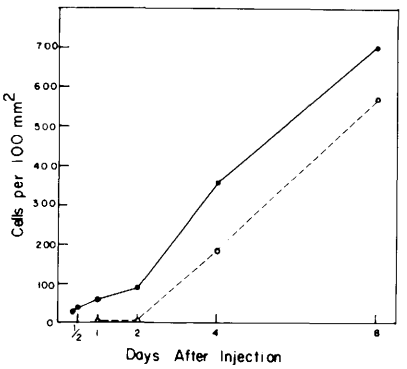

FIG. 1. Antibody-containing cells in the mouse spleen after a primary intravenous injection of HGG. Each point represents the mean cell count in the spleens of 3 mice. ●——●, total antibody-containing cell number at each time; ○-----○, antibody-containing cell number in the white pulp.

Sections stained for mouse globulin (anti-MGG) revealed many more fluorescent cells in the red pulp of the mouse spleen at the early stage of the primary response than did the FITC-HGG conjugate. The MGG-containing cells were observed in the arteriolar areas, the subcapsular areas, and the areas near the edges of the follicles. Most of them, but not all, were found forming small clusters. Scarcely any fluorescent cells were found in the white pulp at this stage. At the later stages of the primary response we found many more fluorescent cells stained with anti-MGG-conjugate than with FITC-HGG conjugate. Anti-MGG conjugate revealed occasionally extra- or inter-cellular gamma globulin in paraffin sections.

The secondary response was about 10 times as extensive, and the changes twice as rapid, reaching their peak after 4 or 5 days. Here too the peak was reached in the white pulp, but the red pulp also contained more cells than during a primary response (Table I).

The exact location of the antibody-containing cells was not always easily assigned, because the observations were made under the fluorescent microscope with dark field illumination. However, the early cells were found in the splenic red pulp near the trabeculae. In the white pulp they appeared in the tissue sheathing the central artery and in the marginal zones around the follicles.

Although many sections were exposed to rhodamine-labeled antiBGz, no antigen remaining from injection was observed.

Table II presents cell counts for primary responses to both BGz and KLH. They also reflect the responses to various doses of BGz and KLH. It is of interest to note the coincidence that the optimal dose for BGz (molecular weight 540,000 daltons) was 50 μg, and that for KLH (7,000,000 daltons) 10 mg, a roughly proportional number of molecules.

Discussion. The first change observed was the appearance of isolated large antibody containing cells 8 hr after the injection of HGG. These cells probably synthesize IgM as Hanaoka, Nomoto, and Waksman have demonstrated in the appendix of the rabbit (11).

The antibody-containing cells always appeared first in the red pulp before spreading into the white pulp. Positive cells appeared in the white pulp only if the dose of antigen was above a threshold which seemed to be different for various antigens. The injection of 1 μg BGz produced no response in the white pulp at any time but 10 μg elicited a measurable response by Day 8. For KLH, however, more than 100 μg was required before any positive cells could be seen in the white pulp. The primary response began slowly, being barely detectable during the first two days, but then increasing from Days 2 to 4. And although there was a doubling of the small number of specific cells in the red pulp, those in the white pulp increased 20-fold during the same 2-day interval. Then, during the next 4 days, the increase slowed in the red pulp but continued in the white pulp. This type of distribu-

TABLE I. PRIMARY AND SECONDARY RESPONSES TO BGz.[a]

BGz (days after injection)	Fluorescent cells/10 mm²		Estimated mean/10^6 cells	W/R[b]
	R	W		
1°[c] 2	4.6	0.3	48	0.07
5	60	8.2	682	0.14
12	33	68	1017	2.1
2°[d] 2	31	30	609	0.95
5	366	673	10060	1.83
8	102	80	1820	0.78

[a] Three mice per group.

[b] W/R is the ratio of the number of cells in the white pulp to those in the red pulp.

[c] 0.1 mg heated BGz intraperitoneally.

[d] 0.1 mg heated BGz in Freund's adjuvant, boosted 4 weeks later with 0.1 mg ip without adjuvant.

TABLE II. DOSE RESPONSES TO A FIRST INJECTION.

Dose (mg)	8 days BGz				12 days KLH			
	Fluorescent cells/10 mm²				Fluorescent cells/10 mm²			
	(R)	(W)	(W/R)	(Total/10⁶)	(R)	(W)	(W/R)	(Total/10⁶)
0.01	19.1	21.4	1.12	405				
0.05	22.1	142.1	6.46	1645				
0.1	14.7	70.7	4.82	854	4.4	0	0	41
0.5	28.8	69.4	2.42	982				
1.0	12.5	30.1	2.40	426	10.2	1.1	0.12	103
5.0	3.7	10.5	2.86	142				
10.0					14.4	31.8	2.2	463

tion of active cells occurred only if a relatively large dose of antigen was administered (5 mg HGG, 10 mg KLH). With smaller doses, the response in the white pulp appeared later or not at all.

Secondary responses to HGG and BGz antigens went through the same changes at a faster pace; the cells in the white pulp on the second day were 100-fold more numerous in absolute numbers and were equal to those in the red pulp. By the 5th day when the red pulp population had increased 10-fold, the white pulp cells had increased 20-fold. If this represents multiplication rather than recruitment, then the cells multiplied once every 9 hr.

During the primary response, cells positive for MGG appeared as a response to antigenic stimulation in more cells than those which contained specific antibody. This finding has been recorded by a number of observers, whose findings are summarized by Cazanave *et al.* (12). Some of these cells were found by them to contain globulin of the same idiotype as the antibody, without *reactivity* for the antigen, a phenomenon not at present understood; perhaps it represents antibody of very low avidity.

At present it is not possible to understand the meaning of the changes described here, but they are clearly related to the differentiation of antibody-forming cells, and to the microenvironment associated with their development. The spread of antibody formation from the red to the white pulp during the primary antibody response may represent the migration of progeny of the cells from the red to the white pulp, or the recruitment of new cells by antigen as the response proceeds. Or, it might conceivably indicate the diffusion of a substance produced by the early cells which triggers the response of the later ones.

Summary. Enumeration of the cells in the mouse spleen making a specific response to antigenic stimulation indicates that the first detectable antibody appears in a few cells in the red pulp in the first 12 hr; later, such cells appear in the white pulp, and, though their number increases in both locations, the gain is more rapid in the white pulp, reaching a peak on the 12th day in the primary response, and on the 5th in the secondary, when there are two to four times as many cells there as in the red pulp. The response in the white pulp requires a higher threshold dose than that in the red pulp.

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