Antigen Binding and the Immune Response
II. The Large Number of Antigen-Binding Cells in Primed Animals
(35061)

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Antigen-sensitive cells are considered to be specific by virtue of their receptors for antigen recognition (1, 2). The differences between primary and secondary responsiveness may be solely quantitative and involve a different number of antigen-sensitive units (3, 4) or may include qualitative components (5–7).

In initiating a direct study of the frequency and receptor content of cells from primed animals, we have examined the binding of beta-galactosidase (BGz) by spleens from previously injected mice. We employed a highly sensitive technique which offers the possibility of measuring a very few molecules of cell-bound antibody (8). This method has been called the A–H assay (antigen binding to cells determined by enzymatic fluorogenic group hydrolysis) and has been reported previously (9, 10).

We have found that 4 months after a primary injection of BGz, although mice were able to respond to a second dose of antigen with characteristic anamnestic vitality, no antibody-producing cells could be detected by the immunofluorescence technique and their serum was indistinguishable from that of uninjected controls. No cell with the capability of binding an unusally large amount of antigen was present in the 25,000 cells tested. However, many cells bound small amounts of antigen, distinguishing this population as a whole from a normal one.

Materials and Methods. Antigen. BGz from *Escherichia coli* ML 308 was kindly supplied by Dr. I. Zabin, Department of Biological Chemistry, University of California, Los Angeles.

Animals. Male and female A/Jax mice were used in these experiments. They were first injected at 3 to 4 months of age.

Immunization. Unless otherwise mentioned, 100 μg of soluble BGz was injected intraperitoneally.

Antigen-binding by cells. The A–H assay described previously (10, 11) was used throughout and is schematically represented in Fig. 1.

A–H cytophilic assay. (a) *in vitro.* This is an application of the A–H assay, used to measure the cytophilic antibody of serum or other fluids (11). It is similar to the procedure of Boyden and Sorkin (12). In these experiments, cells from the spleens of normal mice were incubated at 0° for 1 hr with the serum to be tested for cytophilic antibody. After washing the cells three times with 1 ml of Minimal Eagle's Medium, BGz was added to a final concentration of 50 μg/ml. The cells were then processed according to the procedure outlined for the A–H assay.

(b) *in vivo.* To determine the amount of cytophilic antibody adsorbed to the cells *in vivo,* spleen cells of immunized animals were processed according to the A–H scheme and droplets were made containing only a few cells (10 to 20). We could discount the occasional droplet with an active cell in it. Heightened binding activity for BGz by the rest of the droplets, over that obtained with normal cells, was attributed to *in vivo*-adsorbed cytophilic antibody. This activity was then expressed in antibody units per cell.

Antibody unit. The unit of antibody is defined as that amount which will bind

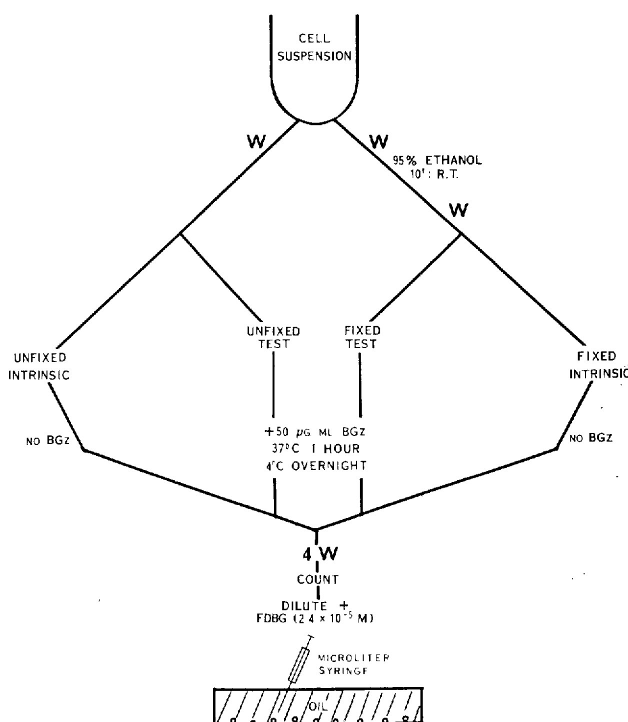

FIG. 1. A–H assay. In this representation, a typical protocol is shown for determination of antigen binding by a cell suspension. 4W = 4 washes with 40 ml of Hanks'; FDBG = fluorogenic substrate, fluorescein-di-beta-galactoside; R.T. = room temperature.

sufficient enzyme molecules to cleave enough fluorogenic substrate in 1 hr at room temperature to produce a reading equivalent to 1×10^{-11} A on the microphotometer, in droplets of 2×10^{-4} ml, as measured by Rotman's technique (13).

Serum titration. Rotman and Celada (14) discovered that a mutant of *E. coli* (W6101), known to have a missense defect in the *Z* gene, produced an antigenically active, but enzymatic inactive BGz (called "AMEF"). Antibody to BGz could restore enzymatic activity to AMEF.

When extracted from the bacteria, some residual BGz activity is present in AMEF. This activity, unlike the activity of the normal BGz, can be eliminated by incubation at 37° for 30 min. To test the antibody content of a serum, residual BGz activity of AMEF was removed in this way. Then, 0.1 ml of "incubated AMEF" was added to 0.1 ml of a 1:10 dilution of serum and assayed as described by Rotman and Celada (14). The AMEF used in these studies was the generous gift of Dr. Boris Rotman, Department of Biomedical Sciences, Brown University, Providence, Rhode Island.

Results. Normal and long-term primary re-

TABLE I. The A–H Activity of Normal and Primed Mice.

		Antibody units/cell	
	Cells/droplet	Fixed	Unfixed
Unimmunized	2800	736	388
4 months after 1°	3250	4800	1433

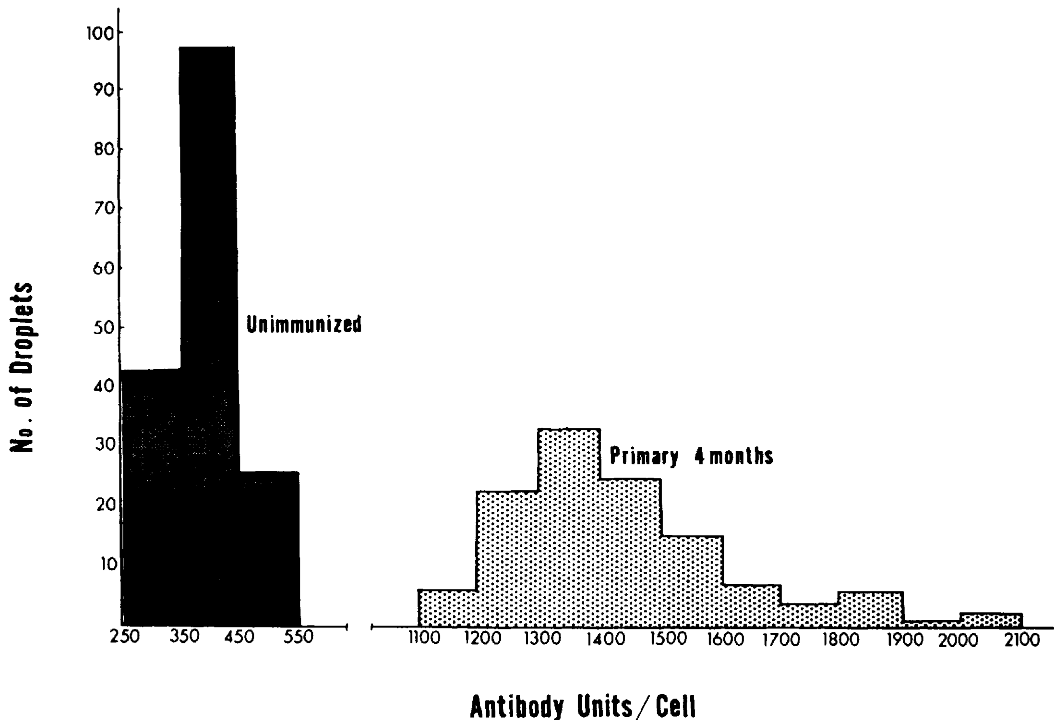

FIG. 2. Distribution of activity of unfixed cells from unimmunized and primed mice. The priming dose injected intraperitoneally, was 100 μg of BGz in saline, 4 months prior to test. Droplets of cells from primed animals contained an average of 220 cells. Droplets of cells from unimmunized animals contained an average of 175 cells.

sponse. The average activities of spleen cells from normal mice and mice primed 4 months earlier, expressed in antibody units per cell, are indicated in Table I. As shown, the primed animals have antigen-binding activities significantly greater than normal.

To determine whether only a few cells were responsible for the activity of primed animals or whether many cells bound a greater amount of antigen than normal, dilutions were made so that each assay droplet contained an average of 220 cells. The activities of hundreds of these droplets as well as of droplets containing cells from unimmunized mice are shown in Fig. 2. In both populations, limited variation of activity may arise from a variation in the number of cells actually present in each droplet. The striking observation, however, is that *all* droplets containing cells from primed animals had activities significantly higher than those from normal ones. Since it was unlikely that so many specific binding cells would exist, we tend to attribute their activity to *in vivo*-adsorbed cytophilic antibody.

Secondary response. Groups of three primed animals were reinjected with 100 μg of soluble BGz intraperitoneally and their cells were tested for antigen-binding activity 5 days later (2°-5d). A sample of these cells was also tested after ethanol fixation; activity distributions for fixed and unfixed cells are shown in Fig. 3. As can be observed, fixed populations showed higher activities. A much broader distribution of values is apparent in Fig. 3, "Fixed." In large part, this may reflect the heterogeneity of antibody levels within the antibody-producing cells of some droplets. The internal antibody is probably not available to bind enzyme in the unfixed

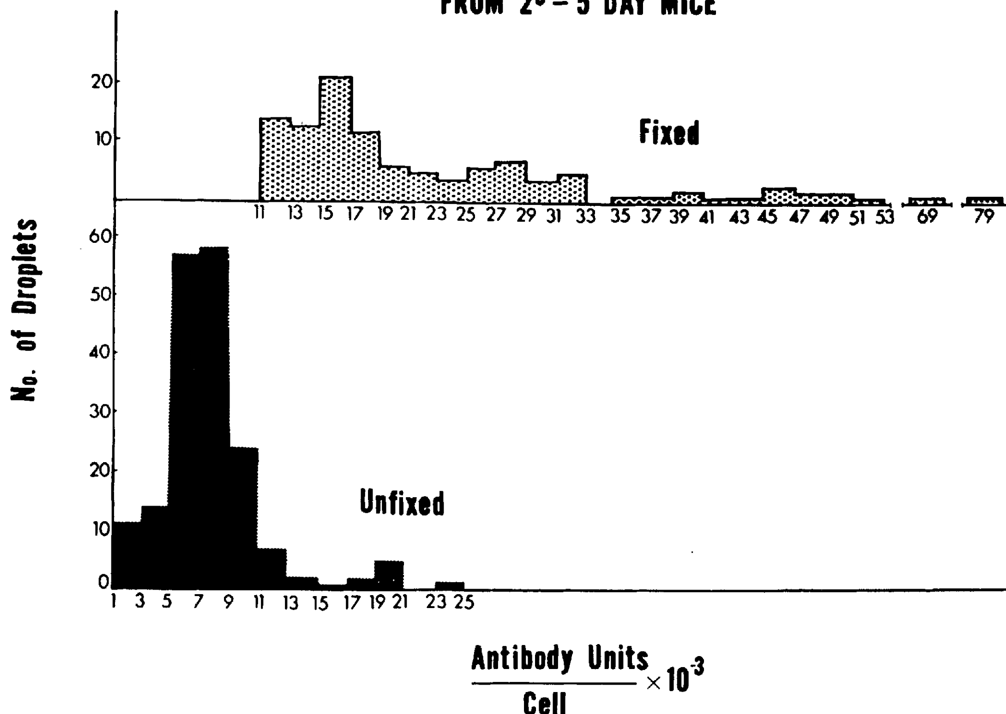

FIG. 3. Distribution of activity of fixed and unfixed spleen cells from mice 5 days after the secondary injection. Doses of 100 μg of BGz in saline were injected intraperitoneally with an interval of 4 months. Droplets of the fixed population contained an average of 105 cells. The unfixed population averaged 235 cells/droplet.

cells. It is thought that fixation may expose some of the internal antibody to BGz, thereby increasing the total BGz binding by the cells.

Primary response. The antigen-binding response following a primary injection of 100 μg of soluble BGz, as measured by the A–H assay, peaks at 4–6 days (9). Distribution studies of spleen cells at the peak of the primary response, 5 days postinjection (1°-5d), are presented in Fig. 4. The most active droplet in the unfixed 1°-5d sample had an activity of 6870 antibody units/cell, a value which is somewhat lower than the mean activity of unfixed cells from 2°-5d animals. Either each active cell in the secondary response binds more BGz than active cells from the primary response, or there is an important contribution by cells whose activity has been passively acquired. It is apparent that for the enumeration of the truly relevant cells in such populations, removal of antibody from nonspecific, passive cells is a prerequisite.

Serum titration. AMEF was used for routine serum titration. To test for the presence of cytophilic antibody, the cytophilic A–H test was employed. Results are shown in Table II. It may be observed that before restimulation, the sera of 4-month-primed mice did not contain antibody detectable by the methods employed. On the other hand, the spleen cells from these animals bound BGz significantly above normal as indicated in column 2 of Table II (under *in vivo* cytophilic).

Discussion. Why is there a difference in the binding activity per cell between unimmunized animals and long-term primed animals? If memory cells (Y) actually exist, which are distinct from unimmunized antigen-sensitive cells (X), the distinction may

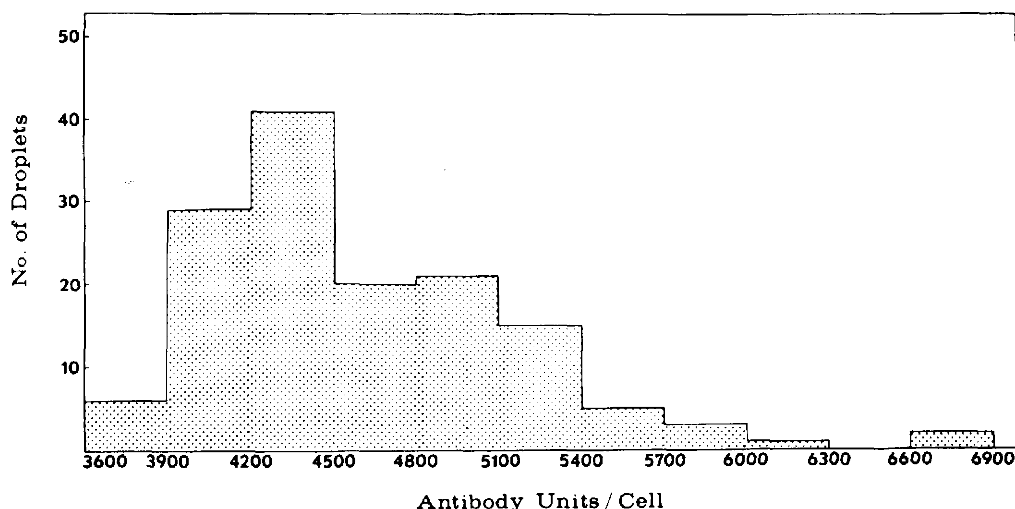

FIG. 4. Distribution of activity of unfixed spleen cells taken 5 days after a primary intraperitoneal injection of 100 μg of BGz in saline. Each droplet contained an average of 295 cells.

be owing to (i) an increased number of antigen-binding receptors per cell, (ii) more effective placement of receptors, or (iii) attainment of independence from obligatory cellular interaction. Only in the first instance would there be a difference in binding activity per cell between late primary and normal animals. Ideally, it should be possible to determine the exact number of antibody molecules on a memory cell.

Our results show that *all* the 220-cell droplets from 4-month-primed mice had activities higher than those from unimmunized animals (Fig. 3). It is extremely unlikely that every droplet in these primed animals would contain either a residual antibody-producing cell or an antigen-sensitive cell. (a) Typical antibody-producing cells can be excluded due to their absence by the criterion of immunofluorescence. (b) If 1% of the 220 cells were true antigen-sensitive Y cells, restimulation with antigen and subsequent division would yield a large proportion of antibody-producing cells. In fact, at the height of the secondary response to sheep erythrocytes, a maximum of only 2% of the cells are active (15, 16); in antiprotein cellular systems, at most 0.8% of the spleen cells are active (17). Apparently, the higher activity in our primed mice must result from a large number of antigen-binding cells which are not necessarily antigen-sensitive cells. This view is substantiated by preliminary experiments with 20-cell droplets taken from animals 4 months after injection with BGz in complete Freund's adjuvant, where we have been able to demonstrate that a majority of droplets have higher activities than unstimulated 20-cell droplets. In addition, cellular studies of Naor and Sulitzeanu (18) of the regional lymph nodes draining sites of BSA injection 100 days earlier have indicated that 23% of

TABLE II. Anti-BGz Determinations.

State of animal	Cell-bound assay; A–H cytophilic[a] *in vivo*	Serum assays: A–H cytophilic[a] *in vitro*	Serum assays: AMEF[b]
Normal	390	410	0.157
1°-5 day	4200	450	0.173
1°-4 month	1200	430	0.158
2°-5 day[c]	5800	4180	1.44

[a] Antibody unit/cell.

[b] mμmoles of o-nitrophenol produced/min/0.01 ml of undiluted serum.

[c] Interval between 1° and 2° injections was 4 months.

all cells bound more antigen than normal cells!

These lines of evidence lead to the conclusion that the binding by a primed cell population is due largely to adsorbed antibody of great longevity which must have adhered to many cells during the initial period of antibody production.

Cytophilic antibody, which is formed by one cell and renders another cell antigen-binding, is well known (19–22). Most authors concur that only macrophages and not lymphocytes can bind cytophilic antibody. However, the existence of antibody cytophilic for lymphocytes which appears during the course of an immune response, is apparent from many reports in which various antigen-binding techniques have been used (18, 23, 24). Likewise, thymus cells are extremely avid cytophilic antibody binders (25). We favor the conclusion that the activity of our primed cells is due to long-lasting cytophilic antibody produced during the immune response [with the reservation that this may not represent the classical cytophilic antibody characterized for a number of species (20)].

We have established that in our population of 25,000 cells, there were no Y cells having an unusually large number of receptors. There may have been some Y cells with a number of receptors which falls within the range of passively acquired binding activities we have found in the primed populations. Were this the case, it would become necessary to make subtle chemical distinctions to differentiate receptors on true memory cells from passively attached cytophilic antibody.

Summary. Four months after a primary injection of beta-galactosidase without adjuvant, washed spleen cells of A/Jax mice bind increased levels of the enzyme *in vitro*, at a time when no antibody activity can be demonstrated in the serum by other criteria. In such mice, a large proportion of spleen cells bear the antigen-binding activity. Hence, not all antigen-binding cells are necessarily antigen-sensitive or antibody-producing cells.

This increased binding can be attributed to cells which have adsorbed cytophilic antibody *in vivo* during the period of antibody biosynthesis.

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