MONOSPECIFICITY OF BONE MARROW-DERIVED LYMPHOCYTES

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The clonal selection hypothesis suggests that during ontogeny individual antibody-forming cell precursors (AFCP), now known to be bone marrow-derived (B) lymphocytes (1), become committed to respond to a limited number of antigens, and express this commitment by displaying immunoglobulin (Ig) receptors on their surface with the same specificity as the antibody that the cell or clone will eventually secrete (2). There is now abundant evidence supporting this hypothesis. Antibody-secreting cells have been shown in general to secrete Ig of a single class, light chain type, allotype, idiotype, and specificity (3). B lymphocytes have been demonstrated to have Ig on their surface (4), and there is much indirect evidence that it serves as receptor for antigen (5). The ability of anti-idiotype antibody treatment of normal spleen cells to specifically inhibit the subsequent production of antibody expressing the idiotype (6) argues strongly that the Ig receptors on AFCP are of the same specificity as the secreted antibody. In addition, AFCP from immune and nonimmune animals have been selectively inactivated by treatment with highly radioactive antigen (7, 8) or selectively removed from cell suspensions by passage over antigen-coated beads (9). Although, taken together, all of this evidence indicates that antibody-secreting cells are monospecific and that AFCP have Ig receptors of restricted specificity, they do not establish whether AFCP are monospecific (having receptors with identical combining sites) or oligospecific (having various receptors with different combining sites) (10). The experiments reported here provide direct evidence for monospecificity of normal and primed B lymphocytes.

We have made use of the observation that multivalent anti-Ig antibody or antigen, reacting with surface Ig receptors on B cells, can induce a redistribution of the Ig molecules, causing them to passively aggregate into patches and actively localize over one pole of the cell, forming a “cap” (11, 12). By allowing

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1 Abbreviations used in this paper: AFCP, antibody-forming cell precursors; B lymphocyte, bone marrow-derived lymphocyte; Fab anti-Mlg, monovalent Fab fragments of rabbit anti-mouse-Ig antibody; Fl, fluorescein (isothiocyanate); G anti-Ri, goat antirabbit-Ig antibody; Ig, immunoglobulin; IgV, Ig variable region genes; POL, polymerized flagellin; R anti-Mlg, purified rabbit antimouse-Ig antibody; R anti-POL, rabbit anti-POL antibody; Rd, tetramethylrhodamine (isothiocyanate); T cells, thymus-derived lymphocytes; VBS, 0.1% bovine serum albumin in Veronal-buffered saline; VBS-azide, VBS + 0.2% sodium azide.
antigen to cap the Ig receptors on antigen-binding B cells, and then separately identifying the cell-bound antigen and surface Ig molecules under noncapping conditions (i.e. in the presence of sodium azide in the cold) (11, 12) using antibodies labeled with two different fluorochromes, it has been possible to estimate how much Ig is left behind when all of the visible antigen-receptor complexes are located in a cap. Our finding that antigen can cap more than 95% of the surface Ig on capped antigen-binding B cells from immunized and unimmunized animals suggests that all of the receptors on such cells are specific for the antigen.

**Materials and Methods**

Polymerized flagellin (POL) (13), IgG fractions of rabbit antimouse Ig and its univalent Fab fragments (Fab anti-MIg), and goat antirabbit-Ig (G anti-Rig) were prepared as previously described (4, 11). Purified rabbit antimouse Ig (R anti-MIg) was prepared by affinity chromatography using a Sepharose column with covalently linked normal mouse IgG (14). Anti-POL serum was raised by immunizing rabbits subcutaneously with 500 μg of POL of *Salmonella adelaide* in complete Freund’s adjuvant, followed by monthly intravenous injections of 100 μg in saline. The rabbits were bled 1–2 wk after each boost. All sera were pooled, and the IgG fraction (R anti-POL) and its Fab fragments (Fab anti-POL) were prepared. The various anti-Ig and anti-POL antibodies and Fab fragments were conjugated with fluorescein isothiocyanate (Fl) (15) or with tetramethylrhodamine isothiocyanate (Rd) (16). Fluoresceinated anti-θAKR (anti-θAKR-Fl) was the same as that recently described (17).

Spleens from normal or immunized (100 μg POL in saline intraperitoneally 4–7 days previously) 2–5-mo old BALB/c or AKR mice were teased in 0.1% bovine serum albumin in Veronal-buffered saline (VBS). After passage through a short column of glass wool, cells were washed and 0.2 ml (10⁶ cells/ml) were incubated with 0.2 ml of POL of *Salmonella derby* at a final concentration of 26 or 13 μg/ml for 15 min at 20°C and then 15 min at 37°C. The cells were then washed and incubated for 10 min at 20°C in 2 ml of VBS plus 0.2% sodium azide (VBS-azide) and then chilled. All subsequent incubations (30 min) and washes were done at 0–4°C and in the presence of 0.2% azide to prevent any further capping of Ig receptors (11, 12). The cells were suspended in 0.02 ml of R anti-POL-Rd (1 mg/ml) to rhodamine label the cells that had bound POL. After washing, the cells were resuspended in 0.02 ml of purified R anti-MIg-Fl or Fab anti-MIg-Fl (5 mg/ml) in order to fluorescein label the Ig receptors, without inducing further capping. In some experiments, cells were treated with an additional layer of G anti-Rig-Fl (0.02 ml of 5 mg/ml) to increase the sensitivity of detection of Ig receptors. In one experiment, spleen cells from immunized AKR mice that had been treated with POL and then R anti-POL-Rd were exposed to 0.02 ml of anti-θAKR-Fl (10 mg/ml) in order to fluorescein label thymus-derived (T) cells. After washing, cells were examined under cover slip in the presence of azide using a Vickers M41 Photoplan fluorescence microscope (Vickers Ltd., Croydon, England) equipped with an HBO-200 mercury lamp, incident illumination and dichroic mirrors, and which was modified to separate fluorescein (BG12 + UG2 primary filters + Ilford 108 barrier filter) and rhodamine (BG12 primary filter + Barr and Stroud interference filter for 556 nm + Wratten 32 barrier filter) excitation, and to allow rapid switching between fluorescence and phase-contrast illumination. POL-binding cells were identified by scanning for rhodamine staining, then examined for anti-Ig fluorescein labeling, and finally observed by phase contrast. Only cells showing surface rhodamine fluorescence were counted as POL-binding cells. Between 1 and 4% of spleen...
cells contained small amounts of intracellular rhodamine without surface staining; these cells did not have detectable surface Ig or θ and the majority took up 1 μm polystyrene beads. These were probably macrophages and granulocytes and were easily distinguished from antigen-binding lymphocytes. Treatment with azide at 0–4°C was apparently unable to prevent completely endocytosis of antigen-antibody complexes by these cells.

To determine the sensitivity of our assay for detecting surface Ig (distributed as a ring), purified R anti-MIg-F1 was titrated in VBS-azide at 0°C by preparing doubling dilutions in equal volumes of the same conjugate that had been “quenched” under ultraviolet light for 12 h so that it was no longer fluorescent but maintained its binding activity. In this way the total number of anti-Ig molecules bound to B cells remained constant but the number of bound fluorescent anti-Ig molecules would be expected to be halved at each dilution. Although the intensity of fluorescence decreased at each dilution, the percentage of fluorescent spleen cells (50–60%) remained unchanged out to a dilution of 1:8 and began to fall at 1:16, indicating that the purified R anti-MIg-F1, under the conditions of our experiments, could detect B cells with 13% (or less) of the normal amount of surface Ig. In experiments where an additional layer of G anti-RIG-F1 was added, which increased the sensitivity of detection about four times, about 97% of the surface Ig would have to be removed from a B cell before Ig was no longer detectable.

RESULTS

The results of typical experiments are shown in Table I. When POL was omitted, no rhodamine staining was seen with R anti-POL-Rd (exp. 1). In spleens from immune mice 0.03–0.1% of nucleated cells bound POL, while the proportion was 0.004–0.007% in normal spleens. More than 95% of POL-binding cells also labeled with purified R anti-MIg-F1 and were thus probably B cells. This was confirmed by the finding that < 5% of POL-binding cells from immune AKR spleens labeled with anti-θAKR-F1 (exp. 5), a reagent that has been shown to specifically stain AKR T cells (17) and that labeled 40% of the nucleated spleen cells in this experiment. In addition, only one POL-binding cell was found among 100,000 thymus lymphocytes taken from hydrocortisone-treated mice (exp. 8). It has been shown that such thymus cells are greatly enriched in immunocompetent lymphocytes compared with normal thymus cells (18). The single POL-binding cell in the thymus stained with R anti-MIg-F1 and may thus have been a contaminating B cell. POL binding was completely inhibited when immune or nonimmune spleen cells were pretreated with purified R anti-MIg at 50 μg/ml for 15 min (exp. 6).

When cells were incubated with POL in the cold and/or in azide, all of the POL-binding cells showed a ring distribution of the rhodamine staining (exp. 2 a). However, when the incubation with POL was carried out at 20° and 37°C, in 55–90% of the POL-binding cells (exp. 2 b–g), the rhodamine labeling was confined to one pole of the cell, indicating that the POL had capped the POL-binding receptors (Fig. 1 a). The remaining labeled cells showed patchy ring rhodamine staining. In all cells showing a ring distribution of rhodamine, the fluorescein staining with R anti-MIg-F1 (or Fab anti-MIg-F1) was also distributed in a ring, as was the case for all the other surface Ig-positive B cells that did not bind POL. On the other hand, in the majority (70–90%) of POL-binding cells show-
### TABLE I

#### Results of POL-Binding Studies with Immune and Nonimmune Spleen Cells

| Experiment no. | Time after immunization | POL-concentration (mg/ml) | R anti-POL-Rd | R anti-M1g-F1 | Additional treatment | POL-binding cells no./10⁵ | Caps* | Corresponding (coincident) caps | % | % |
|----------------|-------------------------|---------------------------|---------------|---------------|----------------------|--------------------------|------|----------------------------------|----|----|
| 1a             | Unimmunized             | 0                         | +            | +             | None                 | 0/200,000                | 0    | 0                                |    | 0  |
| 1b             | 4                       | 0                         | +            | +             | None                 | 0/200,000                | 0    | 0                                |    | 0  |
| 2a             | 4                       | 26                        | +            | +             | None                 | 31/45,000                | 70   | 0                                |    | 0  |
| 2b             | Unimmunized             | 26                        | +            | +             | None                 | 17/440,000               | 60   | 62                               |    | 62 |
| 2c             | 26                      | +                         | +            | None          | None                 | 21/326,000               | 56   | 92                               |    | 92 |
| 2d             | 5                       | 26                        | +            | +             | None                 | 15/24,000                | 60   | 76                               |    | 76 |
| 2e             | 6                       | 26                        | +            | +             | None                 | 12/26,000                | 50   | 55                               |    | 55 |
| 2f             | 6                       | 13                        | +            | +             | None                 | 25/91,000                | 70   | 80                               |    | 80 |
| 2g             | 7                       | 26                        | +            | +             | None                 | 7/15,000                 | 50   | 70                               |    | 70 |
| 3a             | Unimmunized             | 26                        | +            | -             | Fab anti-M1g-F1      | 12/200,000               | 6    | 53                               |    | 53 |
| 3b             | 5                       | 26                        | +            | -             | Fab anti-M1g-F1      | 23/300,000               | 80   | 78                               |    | 78 |
| 4a             | Unimmunized             | 26                        | +            | +             | G anti-R1g-F1        | 10/140,000               | 7    | 60                               |    | 60 |
| 4b             | 5                       | 26                        | +            | +             | G anti-R1g-F1        | 27/40,000                | 70   | 80                               |    | 80 |
| 5a             | 4                       | 26                        | +            | -             | Anti-θAKR-F1         | 83/80,000                | 100  | 70                               |    | <5% θ<sup>+</sup> |
| 6a             | Unimmunized             | 26                        | +            | -             | Pretreated with      | 0/200,000                | 0    | 0                                |    | 0  |
| 6b             | 6                       | 26                        | +            | -             | R anti-M1g           | 0/150,000                | 0    | 0                                |    | 0  |
| 7a             | 5                       | 26                        | +            | +             | None                 | 15/24,000                | 60   | 70                               |    | 70 |
| 7b             | 5                       | 26                        | +            | +             | POL plus Fab anti-POL-F1 | 18/30,000              | 60   | 65                               |    | 65 |
| 8              | Unimmunized            | 26                        | +            | +             | None                 | 1/100,000                | 1    | 100                              |    | 100 |

* POL-binding cells were counted as "caps" when rhodamine fluorescence was restricted to ≤½ the cell surface.
† Capped POL-binding cells were considered "corresponding" when all of the fluorescein labeling (with R anti-M1g-F1) coincided with the capped rhodamine staining (with R anti-POL-Rd).
§ Cells were incubated with POL at 0°C in VBS-azide.
¶ Only 2 out of 83 POL-binding cells labeled with fluorescein and even then they were equivocal.
¶* Thymus cells were used from 5-wk old BALB/c mice that had received 2.5 mg of hydrocortisone acetate intraperitoneally 8 h previously; the single POL-binding cell stained with R anti-M1g Fl.

#### Notes:
- A capped distribution of the rhodamine staining, the fluorescein labeling with R anti-M1g-F1 showed a corresponding cap distribution (exps. 2 b-g) with no fluorescence detectable outside the cap (Fig. 1 b). The same high correspondence of rhodamine and fluorescein cap labeling was seen with Fab anti-M1g-F1 (exp. 3) that has been shown not to induce redistribution of Ig (11, 12).
- Even when G anti-R1g-F1 was added after the R anti-M1g-F1 (exp. 4), no fluorescein staining could be seen outside the region of capped POL on the great majority of capped POL-binding cells. In those POL-binding cells where the rhodamine and fluorescein staining did not correspond (less than 10% of all binding cells and 10-30% of capped binding cells), the fluorescein staining was most intense.
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FIG. 1. Demonstration of POL binding and Ig on immune spleen cells exposed to POL under capping conditions and then labeled with R anti-POL-Rd and R anti-MIg-F1 under noncapping conditions. (a) Capped POL-binding cell examined for rhodamine excitation. (b) Same field examined for fluorescein excitation showing capped Ig on the POL-binding cell and ring-distributed Ig on two non-POL-binding cells. Both photographs X 1,000.

at the pole of the rhodamine cap but also could be seen on the rest of the cell surface. To determine if the noncapped Ig on such cells still had anti-POL specificity, cells treated with POL and R anti-POL-Rd were treated in azide at 0°C either with R anti-MIg-F1 or with more POL (26 μg/ml) followed by Fab anti-POL-F1 (10 mg/ml) (exp. 7). In both cases, 25-30% of the POL-binding cells showed rhodamine caps with fluorescein rings, suggesting that most, and possibly all of the noncapped Ig on these cells was specific for POL, but had not bound POL and/or did not cap during the first exposure to POL.

DISCUSSION

These experiments confirm that POL (19), like other antigens with repeating identical determinants (11, 12, 20), can cap receptors specific for the antigen on B lymphocytes. The ability of immunoabsorbent-purified anti-Ig to completely inhibit POL binding, taken together with the ability of POL to cap Ig on POL-binding cells, reinforces the already compelling evidence that the B cell receptors are Ig. The finding of few if any POL-binding T cells may be related to the general (but not universal) difficulty in demonstrating antigen binding to T cells, or to specific properties of some polymeric antigens (discussed in reference 21).

The main purpose of this study was to determine if all of the Ig on an individual B cell has the same specificity. The finding that POL was able to cap all of the Ig detectable by immunofluorescence on most POL-binding cells from normal and immunized mice suggests that this is the case. The validity of this conclusion depends in large part on the sensitivity of immunofluorescence and on the available evidence that when surface molecules are cross-linked by multivalent antibody or ligand and actively redistributed into a cap, other nonbound molecules in general are not trapped nonspecifically and capped in the process. When both R anti-MIg-F1 and G anti-RIg-Fl were used together, the sensitivity of the immunofluorescence detection of surface Ig was such that more than 95% of the Ig on a capped POL-binding cell must have moved into the cap.
in order for the rest of the cell to appear Ig free. It is unlikely that the ability of POL to cap the great majority of Ig molecules on an individual POL-binding cell is due to nonspecific trapping of non-POL-specific Ig, since Ig has been shown to be randomly dispersed on the surface of B cells (22) and there is increasing evidence that separate surface protein molecules cap independently on lymphocytes. For example, Ig has been shown to move independently of H-2 (11) and HL-A alloantigen (23), and of lectin receptors (M. Greaves, personal communication), and the allelic products of the H-2 (24) and HL-A (25) loci recently have been shown to redistribute separately.

The most likely explanation of our findings is that individual virgin and primed B cells display only one type of receptor, implying that only one Ig variable region (V) gene per light or heavy chain is expressed. This in turn suggests that either maternal or paternal IgV genes are expressed in a single B cell but not both, which is compatible with the various demonstrations of allelic exclusion, showing that only one parental Ig allotype is expressed in antibody-secreting cells from animals heterozygous at various Ig genetic loci (reviewed in reference 3). More importantly, our results support the evidence that the principle of allelic exclusion also applies to Ig receptors on AFCP (26).

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