BINDING OF ANTIGEN BY IMMUNOCYTES

II. Effect of Specific Ig on Antigen Binding by MOPC 315 Cells*

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Antibody has been postulated to regulate its production through a feedback mechanism, which modulates the amount of antigen available in immunogenic form (1). However, new antibody synthesis may occur even in the presence of a large excess of circulating antibody, e.g., after secondary immunization or after reduction of serum antibody levels by exchange transfusion or immunoabsor-<ref>
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Walter and Eliza Hall Institute, Melbourne, Australia), were carried by serial transplantation in female BALB/c mice. Single cell suspensions were prepared as previously described (5). Antigens. Dinitrophenylated bovine serum albumin (DNP-BSA)\(^1\) and N-2,4-dinitrophenyl-\(\epsilon\)-amino-N-caproic acid (DNP-EACA) were prepared as previously described (5, 8). There were approximately 23 DNP groups per molecule of DNP-BSA based upon its absorbancy at 360 nm when \(\epsilon = 17,500\) for DNP-lysine. DNP-BSA was radiolabeled by lactoperoxidase-catalyzed iodination (9) to a sp act of 5-10 \(\times 10^4\) cpm/\(\mu\)g. Tritiated DNP-EACA was prepared by the reaction of \(^1\text{H}\text{-}1\)-fluoro-2,4-dinitrobenzene (DNFB) (Amersham/Searle Corp., Arlington Heights, Ill.) with EACA (5, 10). Sp act was 2 \(\times 10^5\) cpm/\(\mu\)g.

Antisera. MOPC 315 serum was obtained from BALB/c female mice 3 wk after the inoculation of \(10^8\) viable MOPC 315 cells. The number of binding sites for DNP in this serum was 2 \(\times 10^8\)/ml by equilibrium dialysis (performed by Dr. Young Tai Kim, Department of Medicine, Cornell University Medical College, New York). Control mouse serum was collected from normal female BALB/c mice. Antiserum to BSA was prepared by four weekly subcutaneous injections of 0.5 mg of purified BSA into guinea pigs.

Binding of DNP Conjugates to Cells. Duplicate or triplicate 0.1-ml aliquots of undiluted normal mouse serum or of serial dilutions of MOPC 315 serum in normal mouse serum were incubated for 2 min with \(^{125}\text{I}\)DNP-BSA (2 \(\times 10^{-12}\) mol) or with \(^1\text{H}\)DNP-EACA (4 \(\times 10^{-11}\) mol) in a final vol brought up to 0.6 ml with phosphate-buffered saline (PBS). 8 \(\times 10^5\) MOPC 315 or HP 76 cells in 0.1 ml of PBS were added and incubated for 30 min at room temperature. \(^{125}\text{I}\)DNP-BSA binding was studied in the presence of a final concentration of 5% BSA (Pentex, Miles Laboratories, Inc., Kankakee, Ill.). Cells were washed with 2 ml of PBS three times for DNP-BSA-binding studies, and once for studies with DNP-EACA.

Cell-bound radioactivity was measured as previously described (5), and the amount of cell-bound DNP conjugate was calculated from its specific activity. Specific binding was calculated by subtracting from the amount of DNP conjugate bound to MOPC 315 cells that bound nonspecifically to control murine myeloma cells (HP-76).

Results

Effect of Specific Ig on Binding of \(^{125}\text{I}\)DNP-BSA to Cells. To determine the effect of specific Ig on the binding of a multivalent antigen to immunocytes, the binding of \(^{125}\text{I}\)DNP-BSA to MOPC 315 or control cells was studied in the presence of increasing concentrations of MOPC 315 or normal mouse serum. The results of a representative experiment are shown in Fig. 1. In the presence of normal mouse serum, MOPC 315 cells bound approximately 10 times more \(^{125}\text{I}\)DNP-BSA than did control cells. High concentrations of MOPC 315 serum inhibited total and specific binding of the conjugate to MOPC 315 cells. The highest concentration of MOPC 315 serum inhibited specific binding by over 90%. Similar findings were obtained in two additional experiments. MOPC 315 serum increased binding of DNP-BSA to control cells and, at low concentrations, to MOPC 315 cells. Low concentrations of MOPC 315 serum increased binding of DNP-BSA to control cells in all of three experiments, and to MOPC 315 cells in four of five experiments. Though the percentage increase in \(^{125}\text{I}\)DNP-BSA binding caused by MOPC 315 serum was greater with control cells, the absolute increase in the average amount of this antigen bound to each cell type was very similar: 2.38 \(\times 10^{-21}\) mol/MOPC 315 cell and 2.13 \(\times 10^{-21}\) mol/HP 76 cell.

Quantitative Relationship between Antigen Binding to Cells and Concentration of Free Ig, Antigen, and Cell Surface Binding Sites. Fig. 2 illustrates the

\(^1\)Abbreviations used in this paper: BSA, bovine serum albumin; DNFB, 1-fluoro-2,4-dinitrobenzene; DNP-BSA, dinitrophenylated bovine serum albumin; EACA, \(\epsilon\)-amino-N-caproic acid; PBS, phosphate-buffered saline.
FIG. 1. Effect of MOPC 315 or normal mouse sera on binding of [125I]DNP-BSA to MOPC 315 or control cells. Fixed amounts of the conjugate (2 × 10^{-12} mol) were incubated with increasing concentrations of MOPC 315 or normal mouse serum for 2 min and with a similar number of MOPC 315 or control cells (8 × 10^6) for 30 min. In this experiment there was no augmentation of [125I]DNP-BSA binding to MOPC 315 cells in the presence of low concentrations of MOPC 315 serum.

ability of cell surface DNP-binding sites to compete with DNP-binding sites on free Ig for DNP-BSA. The data were calculated from the average of the experiments described above, and from earlier studies indicating that there were 8.4 × 10^4 binding sites for DNP-BSA per MOPC 315 cell (5) and 2 × 10^{16} binding sites for DNP per ml of undiluted MOPC 315 serum. The concentration of DNP-BSA used bound approximately 30% of available cell surface binding sites for this conjugate. 90 binding sites on free Ig were required per cell surface
binding site to inhibit by 50% specific binding of the conjugate to cells. Specific binding of conjugate to cells still occurred with a 30,000-fold excess of free to cell surface binding sites. On the basis of two binding sites per MOPC 315 IgA molecule, \(3.8 \times 10^8\) Ig molecules per cell were required for 50% inhibition of DNP-BSA binding and specific binding still occurred with an excess of \(1.25 \times 10^9\) Ig molecules per MOPC 315 cell. Thus, cells can compete very effectively with serum Ig for antigen and can specifically bind significant quantities of antigen despite the presence of a large excess of free specific Ig.

**Effect of Anticarrier Antibody on Binding of DNP-BSA to Cells.** Since hapten-specific Ig can increase binding of DNP-BSA to control or MOPC 315 cells, studies were conducted to investigate the effect of antibody to carrier. A fixed amount of \(\text{[125I]}\)DNP-BSA \((2 \times 10^{-12} \text{ mol})\) was mixed with increasing concentrations of guinea pig anti-BSA serum or MOPC 315 serum for 2 min and with a similar number of MOPC 315 or control cells for 30 min. In two such experiments, anti-BSA serum consistently augmented by 20-90% total binding of \(\text{[125I]}\)DNP-BSA to control cells, but did not augment binding to MOPC 315 cells. As a consequence, anti-BSA serum slightly decreased (approximately 15%) the calculated specific binding to MOPC 315 cells. The increased amount of conjugate bound to control cells in the presence of anti-BSA serum averaged \(2.2 \times 10^{-21} \text{ mol}\) per HP 76 cell, an increase similar to that occurring with MOPC 315 serum.

**Effect of Time of Incubation.** Earlier studies have shown that specific binding of DNP conjugates to MOPC 315 cells increases with time of incubation (5). The effect of this variable on antigen binding to these cells in the presence of specific Ig was tested. 0.5 ml of \(\text{[125I]}\)DNP-BSA \((5 \times 10^{-12} \text{ mol})\) was preincubated with 0.5 ml of undiluted MOPC 315 or normal mouse serum for 30 min to mimic the in vivo situation where antigen most likely interacts with antibody before reacting with cells. 12 million MOPC 315 or control myeloma cells were added in 3 ml of PBS, and duplicate 0.2-ml aliquots of the resulting suspension assayed at several time intervals thereafter. Under the conditions of this experiment, the ratio of free to cell surface binding sites was 10,000 to 1. As can be seen in Table I, specific binding of \(\text{[125I]}\)DNP-BSA to MOPC 315 cells occurred within 30 s in the presence of normal mouse serum, and increased thereafter. MOPC 315 serum completely inhibited specific binding of the conjugate initially, but specific binding did occur after 30 min and increased slowly thereafter. Thus a prolonged period of time is required to establish equilibrium.

**Effect of Specific Ig on Binding of Univalent Conjugate to Cells.** The effect of specific Ig on the binding of the univalent conjugate \(\text{[3H]}\)DNP-EACA to MOPC 315 cells was determined as described above with \(\text{[125I]}\)DNP-BSA, except that BSA was omitted and the cells were washed only once with PBS. Prior studies had indicated these modifications were necessary to obtain optimal specific binding of this ligand to MOPC 315 cells (5). The result of a representative experiment is shown in Fig. 3. In the presence of normal mouse serum, MOPC 315 cells bound approximately three times more \(\text{[3H]}\)DNP-EACA than did control cells. Increasing concentrations of MOPC 315 serum decreased total binding to both MOPC 315 and control cells. However, binding to MOPC 315 cells was decreased to a greater extent than to control cells. At the highest serum
TABLE I
Effect of Incubation Time on Binding of DNP-BSA to MOPC 315 Cells in Presence of Specific Ig

| Sera* | Cells† | Amount of \([^{125}I]\)DNP-BSA (10⁻¹⁴ moles) bound to cells§ |
|-------|--------|----------------------------------------------------------|
|       |        | Total          | Specific[] |
|       | 0.5 min | 5 min | 30 min | 24 h | 0.5 min | 5 min | 30 min | 24 h |
| MOPC 315 | MOPC 315 | 0.14 | 0.17 | 0.40 | 0.54 | 0 | 0 | 0.17 | 0.26 |
| MOPC 315 | HP 76 | 0.18 | 0.17 | 0.23 | 0.28 | | | |
| Normal | MOPC 315 | 0.54 | 0.75 | 1.42 | 2.09 | 0.37 | 0.53 | 1.19 | 1.74 |
| Normal | HP 76 | 0.17 | 0.22 | 0.23 | 0.35 | | | |

* 0.5 ml undiluted.
† 1.2 x 10⁷ cells.
§ Average amount \([^{125}I]\)DNP-BSA bound to duplicate aliquots of 6 x 10⁶ cells incubated with 5 x 10⁻¹² mol of the conjugate in 4 ml PBS.
Calculation by subtracting moles \([^{125}I]\)DNP-BSA bound to HP 76 cells from amount \([^{125}I]\)DNP-BSA bound to MOPC 315 cells.

Fig. 3. Effect of MOPC 315 or normal serum on binding of \([^3H]\)DNP-EACA to MOPC 315 or control cells. Fixed amounts of \([^3H]\)DNP-EACA (4 x 10⁻¹¹ mol) were incubated with increasing amounts of MOPC 315 or normal mouse serum for 2 min, and with a similar number of MOPC 315 or control cells (8 x 10⁵) for 30 min.

Concentration, specific binding was inhibited by 74%. No consistent enhancement of total or specific binding to MOPC 315 or control cells was observed at any dilution of MOPC 315 serum.

Fig. 4 illustrates the ability of cell surface DNP-binding sites to compete with DNP-binding sites on free Ig for DNP-EACA. On the basis of there being 1.1 x 10⁷ binding sites for DNP-EACA per MOPC 315 cell and the amount of DNP-EACA and MOPC 315 serum used, 40 binding sites on free Ig were required per cell surface site to inhibit specific binding of DNP-EACA by 50%.
FIG. 4. Inhibition of specific binding of [\textsuperscript{3}H]DNP-EACA to MOPC 315 cells by increasing ratio of free to cell surface DNP-binding sites. Inhibition is calculated as in Fig. 2.

Discussion

The main finding of this study is that immune cells can specifically bind and retain antigen in the presence of a large excess of specific Ig. This finding provides a partial explanation for the ability of a small amount of antigen to stimulate immunocytes in the presence of excess antibody as occurs in secondary immunization, in the persistent antibody response to slowly catabolized antigen (11), or after the partial depletion of circulating antibody (2-4).

In the past we have suggested that antibody regulates its own synthesis through a feedback mechanism, the central feature of which is a dynamic equilibrium between circulating antibody and persisting immunogen (1). In the presence of large amounts of antibody, which late in the immune response will be of high affinity, the quantity of available free immunogen will be very small. Cells must consequently have an advantage over antibody if they are to effectively interact with antigen. B-cell binding of antigen may be favored by a number of mechanisms including helper T cells, presentation of antigen on macrophage surface, and antigen concentration in specific anatomical areas of lymphoid organs (12). In addition, cells having multiple binding sites and thus an effective valence probably much greater than that of circulating antibody, may be able to bind antigen more avidly than antibody. Theoretical considerations (13) and in vitro experiments (14-18) indicate that multiple bond formation causes a marked enhancement of binding energy. We have confirmed that these considerations apply to the binding of antigen by cells. By direct measurement, the binding affinity of polyclonal antigen to MOPC 315 cells is 100-300 times greater than that of univalent antigen of similar specificity (5).

This study allows the competition between specific free Ig and immunocytes for specific antigen to be expressed quantitatively. Under the conditions of these experiments, approximately 90 binding sites on free Ig were required per cell surface binding site to inhibit by 50% specific binding of DNP-BSA. Significant specific binding still occurred with a 30,000-fold excess of free Ig per cell surface
binding site or in the presence of $1.25 \times 10^9$ molecules of Ig per MOPC 315 cell. On the basis of reasonable assumptions it is possible to extend these numbers to a biological framework and demonstrate that cells can effectively capture a small quantity of antigen administered to an animal with a high concentration of circulating antibody. If it is assumed that a mouse hyperimmunized to DNP-BSA has an IgG concentration of 20 mg/ml, that 10% of it is specific antibody, and that extravascular IgG concentration is 50% that of serum, then it can be calculated and there will be $3.1 \times 10^{18}$ molecules of IgG per ml of extravascular fluid. If this mouse is boosted with 0.1 mg of DNP-BSA and it is assumed that the antigen is distributed freely throughout all tissues (density assumed to be similar to water: 1 g/ml), then the concentration of the antigen in a 30 g mouse will be 3.3 µg/ml. The concentration of DNP-BSA molecules, based on a mol wt of 84,000, would be $2.43 \times 10^{13}$/ml. The ratio of antibody to antigen molecules will be approximately 100 to 1. This ratio will be lower if, as is probable, DNP-BSA does not enter intracellular spaces. In our experiments the concentration of DNP-BSA was $1.7 \times 10^{12}$ molecules per ml and that of MOPC 315 Ig at which specific antigen binding to cell still occurred $1.4 \times 10^5$ molecules per ml. The ratio of antibody to antigen was approximately 1,000 to 1. Thus, specific antigen binding to cells occurred in vitro at a lower concentration of antigen and in the presence of greater excess of antibody than that estimated to be present in vivo during secondary immunization.

Antigen tends to preferentially localize, and is thus concentrated, in specific anatomic areas of lymphoid organs, whereas extravascular antibody is in equilibrium with serum Ig and hence presumably at a similar concentration throughout the extravascular space. Consequently, the ratio of antibody to antigen at the actual site of antigen interaction with cells will probably be lower than that calculated above, shifting the reaction in favor of antigen binding to cells. The presence in MOPC 315 serum of antibody to myeloma-associated antigens or to idiotypic determinants on cell surface IgA would not alter these conclusions. Indeed, by binding to MOPC 315 cells such antibodies might reduce the effective number of available binding sites for DNP-BSA so that the actual ability of MOPC 315 cells to compete for antigen would be even greater than that observed in these experiments. Nor would the higher binding affinity of antibody formed late in the immune response in comparison to that of MOPC 315 alter these conclusions since it would be matched by a comparable increase in the binding affinity of cell surface binding sites (19). In fact, a discrepancy between the binding affinity of serum antibody and that of binding sites on potential antibody-forming cells may explain the ability of passive antibody to suppress the primary antibody response at serum concentrations which do not inhibit the secondary response (21). Antibody-mediated suppression is most efficient with high affinity antibody (20, 21) which will probably bind antigen more effectively than cells whose binding sites are of low average affinity at the onset of immunization.

Total and specific antigen binding to cell was time dependent. Prolonged incubation permitted specific antigen binding to cells in spite of concentrations of specific Ig which completely inhibited specific binding initially. Thus equilibrium between antigen, circulating antibody, and specific cells is achieved
slowly in vitro, and perhaps not at all in complex and dynamic in vivo systems. The rate of reaching equilibrium may play a critical role in influencing the amount of antigen bound to cells. The effect of free antibody is probably to reduce the effective free antigen concentration and thus to slow the forward rate of the reaction of antigen with cell surface binding sites. It is probable that once antigen interacts with cell surface binding sites multiple bonds are readily formed as a consequence of the mobility of cell surface Ig, and the probability of the antigen-cell complex dissociating becomes very low. Thus, one would predict that if sufficient time were allowed, circulating antibody would actually be very inefficient in blocking binding of multivalent antigens to cells.

After prolonged incubation with anti-DNP Ig and DNP-BSA, MOPC 315 cells still bound considerably more of the conjugate than did control myeloma cells. Furthermore, the rate of increase in DNP-BSA binding was greater to MOPC 315 cells than to control cells. Since both MOPC 315 and control cell suspensions presumably contain a similar proportion of macrophages, these findings make it unlikely that cells coated with cytophilic antibody contribute to the greater binding of DNP-BSA to MOPC 315 as compared to control cells.

Binding of univalent DNP-EACA to cells was inhibited by specific Ig more readily than that of the multivalent conjugate. Strict comparisons cannot be made between these experiments, because they could not be performed under precisely comparable variables. Binding of DNP-BSA and DNP-EACA was studied at similar concentration of cells, antibody, and DNP groups, but the molar concentration of DNP-BSA was 23 times less than that of DNP-EACA (because there were 23 DNP groups per DNP-BSA molecules). Furthermore, under the conditions of these assays, bound DNP-BSA occupied 30% of available binding sites on MOPC 315 cells, whereas DNP-EACA occupied only 7% of available sites. However, our earlier studies have shown that the binding of univalent hapten to cells is markedly weaker than that of multivalent ligands of similar specificity (5). Binding of univalent hapten to cells was so weak, that 90% of it could be blocked by physiological concentrations of proteins. These findings suggest that the arrangement of antigenic determinants into a polyvalent array is necessary to achieve adequate binding of antigen to cells. Thus, the carrier requirement for immune response to hapten may be, in part, to present the hapten in the polyvalent form required for its binding to immunocytes.

These experiments and those of others (5, 22-24) indicate that antigen is capable of binding nonspecifically to a variety of cells, and that binding is augmented by aggregation of the antigen into immune complexes by antibody to the hapten or the carrier. Nonspecific enhancement of antigen binding to cells by small amounts of antibody may provide a helper mechanism that promotes immune responses at low antigen concentration by increasing antigen binding to lymphoid cells and to macrophages. This mechanism would operate in addition to the antigen-concentrating function provided by specific antigen-binding sites on B and T cells, and may be mediated by Ig and complement receptors on B cells and macrophages. This mechanism might be one of the factors contributing to the augmentation of antibody formation by low doses of passive antibody (25-27) and the increased antihapten antibody response resulting from preimmunization with carrier (28-30). Finally increased cellular binding of antigen
presented as immune complexes would be consistent with mediation of T- and B-cell cooperation by carrier-specific antibody, a possibility suggested by Bretscher and Cohn (31) and by Feldmann (32).

Summary

The effect of specific immunoglobulin (Ig) on specific binding of antigen to cells has been studied in a model system consisting of murine myeloma cells (MOPC 315), MOPC 315 serum, and DNP conjugates. MOPC 315 serum, which has IgA specific for DNP, specifically inhibited the binding of DNP conjugates to these cells.

Using this model it was found that cells have a marked advantage over free specific Ig in binding multivalent antigen molecules and retaining them in a bound state. Cells were able to specifically bind multivalent antigen in the presence of a large excess of free specific Ig. The kinetics of antigen binding to cells was slow, and prolongation of time of incubation increased the amount of specific binding. Both antihapten and anticarrier Ig augmented nonspecific binding of multivalent but not of univalent hapten to control cells. Furthermore, antihapten Ig at low concentration increased antigen binding to specific cells.

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