BINDING OF ANTIGEN BY IMMUNOCYTES

I. Effect of Ligand Valence on Binding Affinity of MOPC 315 Cells for DNP Conjugates*

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It has recently been observed that many weeks after injection of small amounts of antigen, removal of antibody from the circulation results in a specific, secondary-type, antibody response (1, 2). This observation suggests that a dynamic equilibrium between persisting immunogen and circulating antibody regulates the immune response (3), and further, that immunocompetent cells can capture antigen in the presence of excess circulating antibody. It has been postulated that immunocompetent cells by virtue of their large number of receptors might have an increased avidity for antigen as compared with an equal number of antibody molecules in solution (4).

The murine plasmacytoma MOPC 315 secretes an IgA which binds dinitrophenyl (DNP)[1] with moderate affinity (5). In the experiments which follow, we describe the use of these myeloma cells as a model in which the interaction of immunoglobulin (Ig) on cell surfaces with antigen can be studied in a homogenous cell preparation. Using this system we have been able to measure the binding affinities of cell surface Ig for univalent and for multivalent DNP conjugates.

Materials and Methods

Source and Preparation of Cells.—The following lines of murine plasmacytomas were carried by serial transplantation in female BALB/c mice (Jackson Laboratory, Bar Harbor,

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[1] *Abbreviations used in this paper:* DNFB, 1-fluro-2,4-dinitrobenzene; DNP, dinitrophenyl; DNP-BSA, dinitrophenylated bovine serum albumin; EACA, ϵ-amino-*N*-caproic acid; PBS, phosphate-buffered saline; SSS, Spinner salt solution.

Maine): (*a*) MOPC 315 cells secreting IgA (obtained from Dr. M. Potter, National Institutes of Health, Bethesda, Md.); (*b*) LPC 1 cells secreting IgG2A (obtained from Dr. F. Hymes, NCI Immunoglobulin Reference Center, Springfield, Va.), and (*c*) HP 76 cells secreting IgM (obtained from Dr. Noel Warner, Hall Institute, Melbourne, Australia). For preparations of cell suspensions, the animals were sacrificed by cervical dislocation 2–4 wk after tumor inoculation, and the tumors were removed and finely minced in Spinner salt solution (Eagle; SSS; Grand Island Biological Co., Grand Island, N. Y.). The cells were passed through a fine stainless steel mesh, washed twice with 40 ml of SSS, and resuspended in 0.5 ml of SSS. Red blood cells and some nonviable tumor cells were lysed by osmotic shock resulting from addition of 4 ml of cold distilled water. After 30 s, physiologic osmolarity was restored by the addition of 40 ml of SSS. Viable cells were pelleted by centrifugation and resuspended to a concentration of 7–10 $\times$ 10^6 per ml in phosphate-buffered saline (PBS; 0.15 M NaCl, 0.01 M potassium phosphate buffer, pH 7.3). Viability ranged from 65 to 85% as judged by trypan blue exclusion.

P_3K murine plasmacytoma cells secreting IgG (obtained from Dr. Kengo Horibata, Salk Institute, San Diego, Calif.) were carried in Dulbecco's medium with 10% horse serum, and were used after washing three times in 40 ml of SSS. Viability of these cells was over 90% as determined by trypan blue exclusion.

Antigens.—Dinitrophenylated bovine serum albumin (DNP-BSA) was prepared by the reaction of 2,4-dinitrobenzene sulfonic acid (Fisher Chemical Co., New York) with BSA (Pentex Biochemical, Kankakee, Ill.) at room temperature under alkaline conditions as described by Eisen, Belman, and Carsten (6). The product was extensively dialyzed against 0.001 M phosphate buffer (pH 7.3) and its concentration determined after drying a known volume to constant weight at 95°C. The preparation used had approximately 23 DNP groups per molecule of BSA based upon its absorbancy at 360 nm taking ϵ = 17,500 for DNP-lysine. DNP-BSA was radiolabeled by lactoperoxidase-catalyzed iodination (7) to a specific activity of 5–10 $\times$ 10^5 cpm/μg.

N-2,4-dinitrophenyl-ϵ-amino-*N*-caproic acid (DNP-EACA) was prepared by the reaction of 1-fluoro-2,4-dinitrobenzene (DNFB; Eastman Organic Chemicals, Rochester, N. Y.) with ϵ-amino-*N*-caproic acid (EACA; Sigma Chemical Co., St. Louis, Mo.) under alkaline conditions. The product was purified by crystallization from hot 50% ethanol. Tritiated DNP-EACA was prepared by the reaction of [^{3}H]DNFB (Amersham-Searle, Arlington Heights, Ill.) with EACA. The product was purified by thin-layer chromatography on silica-gel plates using *p*-dioxane as solvent. Details for preparation of these haptens have been presented previously (8). Specific activity of the product was 2 $\times$ 10^5 cpm/ng. Goat IgG was prepared by salting out with ammonium sulfate, and the dialyzed precipitate was chromatographed on DEAE-Sephadex (A-25).

Antisera.—Antisera to immunoelectrophoretically pure mouse or human IgG were prepared by weekly injection of goats with 0.5 mg of the respective immunoglobulin in complete Freund's adjuvant. Serum was collected after 6–10 injections and was demonstrated to be specific for the immunizing antigen by immunoelectrophoresis. Antisera to mouse IgG reacted against mouse κ-chains.

Binding of DNP Conjugates to Cells.—0.1 ml of myeloma cell suspensions, adjusted to a concentration of 7–10 $\times$ 10^6 cells per ml, were incubated with varying concentrations of DNP conjugate. Incubation was performed at 27°C. Cells were pelleted at 800 *g* for 6 min and were washed repeatedly with 2 ml of PBS as indicated in text. The amount of cell-bound DNP conjugate was calculated from its specific activity and the counts per minute of the washed cell pellet. For ^{3}H counting, the cell pellet was dissolved in 1 ml of NCS (Amersham-Searle) at 37°C for 1 h, and a 0.7 ml aliquant was counted in 10 ml of Liquiflor (New England Nuclear Corp., Boston, Mass.). All assays were performed in duplicate or triplicate tubes.

Specific binding of DNP conjugate to MOPC 315 cells was calculated by subtracting the

amount bound nonspecifically to control murine myeloma cells from the amount bound to a similar number of MOPC 315 cells under identical conditions. Three different lines (P_3K, LPC 1, and HP 76) of murine myeloma cells were used as controls and yielded similar results. The average association constant (K_o) and the number of binding sites per cell were calculated from a Scatchard plot of specific binding of DNP conjugate to MOPC 315 cells.

RESULTS

Binding of [^{125}I]DNP-BSA to Cells.—In order to determine whether cell surface molecules with specificity for DNP are present on MOPC 315 cells, the binding of [^{125}I]DNP-BSA to MOPC 315 was compared with its binding to other myeloma cells whose secreted proteins have no detectable rectivity with DNP. In addition, the effect of [^{125}I]DNP-BSA concentration on the quantity of ligand bound was determined.

As can be seen in Fig. 1, at every ligand concentration studied considerably more [^{125}I]DNP-BSA was bound to MOPC 315 cells than to control cells (P_3K). The ratio of [^{125}I]DNP-BSA bound by MOPC 315 to the amount bound to control cells was greatest at low ligand concentrations. The maximum ratio obtained was 8, but at the highest [^{125}I]DNP-BSA concentration it decreased to 1.7. Similar results were obtained with either HP 76 or LPC 1 myeloma cells as controls.

The greater binding of DNP-BSA by MOPC 315 as compared with control cells could be due to variations in the viability or in the surface area of the different cell types used. Similar results were obtained, however, when control cells of different degrees of viability (by trypan blue exclusion) were employed.

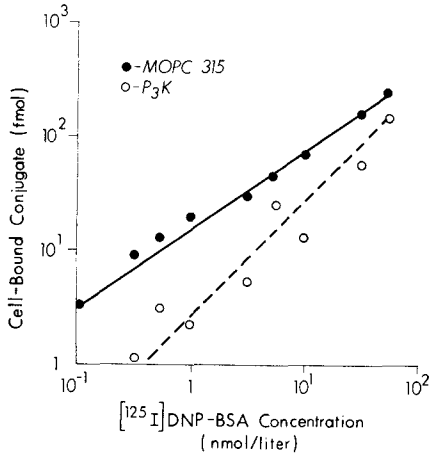

FIG. 1. Binding of [^{125}I]DNP-BSA to cells. Similar numbers of MOPC 315 or P_3K cells were incubated for 10 min with varying concentrations of the conjugate in medium containing 5% BSA. Amount of cell-bound [^{125}I]DNP-BSA was determined after cells had been washed three times with 2 ml of PBS.

Furthermore, when the size of the cells was estimated by measuring the smallest and largest diameter of 10–20 cells of each type, MOPC 315 cells were found to be intermediate in size as compared with the different control cells used. Thus, the preferential binding of [^{125}I]DNP-BSA by MOPC 315 cells cannot be explained by differences in cell size or viability.

Factors Affecting the Binding of [^{125}I]DNP-BSA to Cells.—The effect of the concentration of protein in the medium, of washing, and of time of incubation on the amount of ligand bound to MOPC 315 and to control cells was studied. The effect of protein in the medium was determined by performing binding studies in the presence of BSA. As can be seen in Fig. 2, the quantity of [^{125}I]-DNP-BSA bound to MOPC 315 or to control cells was markedly decreased by increasing the concentration of BSA. It is of note that without BSA nonspecific binding was so great that specific binding was completely obscured. After reducing nonspecific binding by the addition of 0.05 mg/ml of BSA, a difference between the binding to MOPC 315 and control cells became evident. With increasing concentrations of BSA, binding to control cells decreased further but not the binding to MOPC 315. Similar results were obtained when goat IgG was used instead of BSA. Since specific binding was maximal when the cells were incubated with 50 mg/ml of BSA, all subsequent binding studies with [^{125}I]DNP-BSA were performed in the presence of this concentration of BSA.

The effect of repeated washing with PBS on the binding of [^{125}I]DNP-BSA

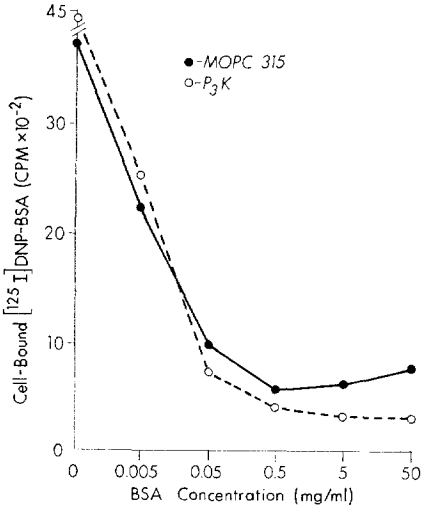

FIG. 2. Effect of inhibiting nonspecific binding of [^{125}I]DNP-BSA with BSA. Similar numbers of MOPC 315 or P_3K cells were incubated with [^{125}I]DNP-BSA (10^{-9} M) for 10 min. Incubation was performed in presence of varying concentration of BSA. Amount of cell-bound [^{125}I]DNP-BSA was determined after cells had been washed three times with 2 ml of PBS.

to cells is illustrated in Fig. 3. There was a marked decrease in the quantity of [^{125}I]DNP-BSA bound to MOPC 315 cells and to control cells as a result of the first two washes. Thereafter, continued washing resulted in a progressive but minimal decrease in bound DNP conjugate. Three washes was selected as the standard procedure since it removed over 90% of [^{125}I]DNP-BSA bound non-specifically to control cells.

The effect of time of incubation on the quantity of DNP conjugate bound to MOPC 315 cells is illustrated in Fig. 4. At room temperature, binding to cells occurred rapidly. Within the first 2 min 60% of the maximum binding to MOPC

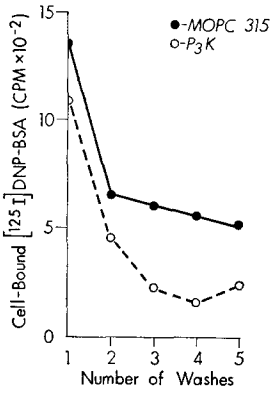

FIG. 3. Effect of washing cells on binding of [^{125}I]DNP-BSA. Similar numbers of MOPC 315 or P_3K cells were incubated with [^{125}I]DNP-BSA for 10 min at 27°C in the presence of 5% BSA, and the quantity of conjugate remaining bound to cells was determined after increasing number of washes with 2 ml of PBS.

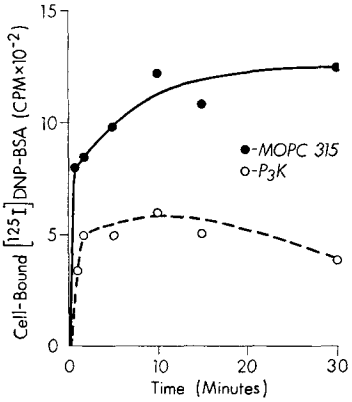

FIG. 4. Effect of time on binding of [^{125}I]DNP-BSA to cells. Similar numbers of MOPC 315 or P_3K cells were incubated with [^{125}I]DNP-BSA (10^{-9} M) in the presence of 5% BSA for varying periods of time. Amount of cell-bound [^{125}I]DNP-BSA was determined after the cells had been washed three times with PBS.

315 cells, and over 90% of maximum binding to control cells had taken place. Maximum binding to control cells took place within 10 min but binding to MOPC 315 cells appeared to increase slowly after the initial rapid phase. All subsequent experiments were performed with 10 min of incubation.

Specificity of Binding of [^{125}I]DNP-BSA to MOPC 315 Cells.—To establish that the binding of [^{125}I]DNP-BSA to MOPC 315 cells was due to the specific interaction of the DNP groups to Ig molecules on the cell surface, attempts were made to inhibit binding with DNP-EACA and with antisera to mouse Ig. As can be seen in Fig. 5, specific binding of [^{125}I]DNP-BSA could be completely inhibited by the univalent ligand, DNP-EACA. On a per-binding-site basis, the smallest ratio of univalent ligand to [^{125}I]DNP-BSA required to com-

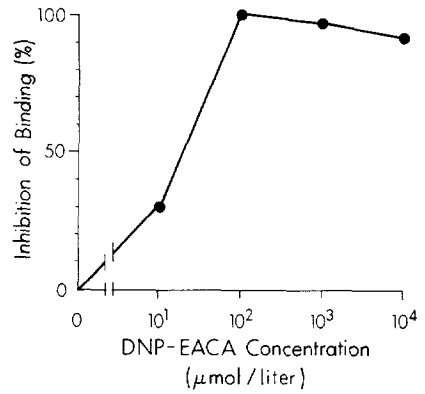

FIG. 5. Inhibition of specific binding of [^{125}I]DNP-BSA to MOPC 315 cells by DNP-EACA. Similar number of MOPC 315 or P_3K cells were incubated with [^{125}I]DNP-BSA (10^{-9} M) in the presence of 5% BSA and increasing concentrations of the univalent ligand DNP-EACA. Inhibition of specific binding was calculated from the formula:

$$\left(1 - \frac{\text{Specific binding to MOPC 315 cells in presence of DNP-EACA}}{\text{Specific binding to MOPC 315 cells in absence of DNP-EACA}}\right) \times 100.$$

pletely inhibit the binding of [^{125}I]DNP-BSA was 500 to 1, based on an estimated 23 DNP groups conjugated to each molecule of BSA.

Specific binding of [^{125}I]DNP-BSA was also completely inhibited by prior incubation of MOPC 315 cells with goat antisera to mouse κ-chains which are present in the IgA protein secreted by MOPC 315 (5). As can be seen in Fig. 6, there is a relationship between the quantity of [^{125}I]DNP-BSA added and the percent inhibition. At the lowest concentration of [^{125}I]DNP-BSA used, specific binding was completely inhibited. If higher concentrations of [^{125}I]DNP-BSA were employed, less inhibition was observed. Under identical conditions goat antihuman IgG had a minimal inhibiting effect on specific binding. These experiments indicate that specific binding of [^{125}I]DNP-BSA to MOPC 315 cells is between the DNP group and specific Ig molecules on the cell surface.

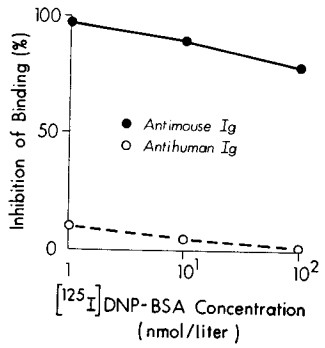

FIG. 6. Inhibition of specific binding of [^{125}I]DNP-BSA by antisera to mouse Ig. Similar number of MOPC 315 or control cells were incubated with 1 ml of goat antimouse Ig or goat antihuman IgG for 10 min at 27°C. After one wash with PBS, cells were incubated with varying concentrations of [^{125}I]DNP-BSA. Inhibition of specific binding was calculated from the formula:

$$\left(1 - \frac{\text{Specific binding to MOPC 315 cells in presence of goat antisera}}{\text{Specific binding to MOPC 315 cells in absence of goat antisera}}\right) \times 100.$$

Association Constant for Binding of [^{125}I]DNP-BSA to MOPC 315 Cells.—The average association constant (K_o) for the interaction of [^{125}I]DNP-BSA to MOPC 315 cells was calculated from the specific binding determined at different concentrations of conjugate. The experiments were conducted under conditions which had been previously determined to maximize specific binding.

A Scatchard plot of the results of a representative experiment is shown in Fig. 7. The average association constant was calculated from the reciprocal of the free hapten concentration at which 50% saturation of binding sites was attained. In four independent experiments the values calculated for K_o were 6.2, 7.2, 7.8, and 13 $\times$ 10^7 L/M (average K_o = 8.5 $\times$ 10^7 L/M). Since DNP-BSA is a multivalent ligand, each molecule could potentially interact with more than one anti-DNP binding site on the MOPC 315 cell. For this reason it was not possible to calculate the exact number of binding sites. However, if it is assumed that the number of binding sites is equal to or greater than the maximum number of DNP-BSA molecules bound per cell, then the minimum number of binding sites detected in the four above experiments averaged 84,000 sites per cell.

Binding of Univalent DNP Conjugate to MOPC 315 Cells.—To evaluate the effect of antigen valence on its binding to cell surface receptors, the binding of the univalent ligand DNP-EACA was compared with that of the multivalent ligand DNP-BSA. Specific binding of [^{3}H]DNP-EACA to MOPC 315 could be demonstrated in experiments similar to those described above using [^{125}I]-DNP-BSA. Fig. 8 illustrates the results of a representative experiment in which the binding of various concentrations of [^{3}H]DNP-EACA to MOPC 315 and

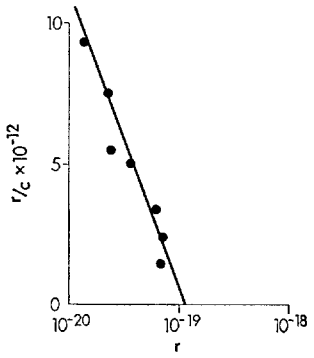

FIG. 7. Scatchard plot of binding of [^{125}I]DNP-BSA to MOPC 315 cells. r is moles of conjugate bound per MOPC 315 cell, and c is the free conjugate concentration. Number of binding sites per cell (n) is calculated by extrapolation of binding curve to its interception with abscissa.

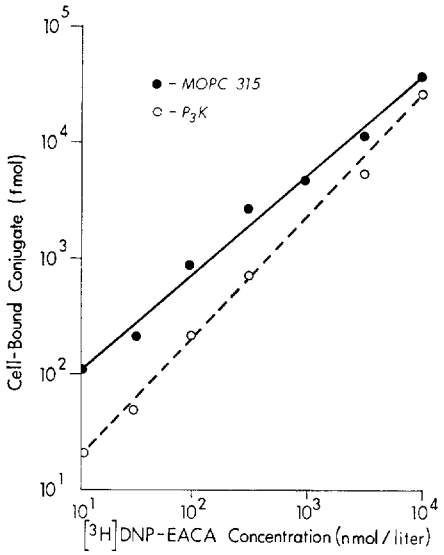

FIG. 8. Binding of [^{3}H]DNP-EACA to MOPC 315 or P_3K cells. Similar numbers of cells were incubated for 10 min at 27°C with varying concentrations of the conjugate. Amount of cell-bound [^{3}H]DNP-EACA was determined after cells had been washed two times with 2 ml of PBS.

to control cells was compared. It can be seen that at every ligand concentration employed, there was more binding to MOPC 315 cells than to control cells. The ratio of moles [^{3}H]DNP-EACA bound by MOPC 315 cells to moles bound to control cells was greatest at low ligand concentration, where a ratio of 5 was observed, and decreased to 1.4 at the highest concentration of ligand used.

Interaction between the DNP conjugate and cells was rapid. 85% of maximal binding occurred within 5 min at 27°C. The rate of the reaction was temperature dependent, only 50% of maximal binding occurring within 5 min at 4°C.

Washing the cells was found to have a profound effect on the specific binding of [^{3}H]DNP-EACA. As can be seen in Fig. 9, the amount of ligand bound to MOPC 315 decreased by 40–90% with each wash cycle. Nonspecific binding of [^{3}H]DNP-EACA to control cells did not decrease significantly after the second wash.

The presence of protein in the incubation mixture was found to decrease greatly both the total and specific binding of [^{3}H]DNP-EACA to MOPC 315.

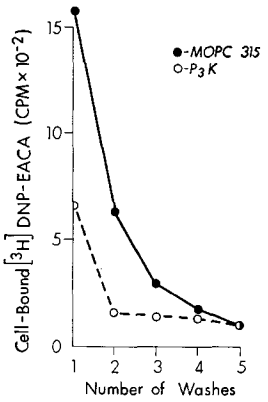

FIG. 9. Effect of washing cells on binding of [^{3}H]DNP-EACA. Similar number of MOPC 315 or P_3K cells were incubated for 10 min at 27°C, and the quantity of conjugate remaining bound to cells was determined after increasing number of washes with 2 ml of PBS.

Specific binding in the presence of 5% BSA was reduced by 85–94%. By contrast, it will be recalled that BSA is required for specific binding of [^{125}I]DNP-BSA to become evident.

Since DNP-EACA is a small molecule, it is possible that its intracellular penetration during incubation falsely elevated the apparent binding of the conjugate to the cell surface. This possibility was made unlikely by the finding that over 93% of the specific binding of [^{3}H]DNP-EACA to MOPC 315 could be inhibited by prior incubation of the cells with goat antimouse Ig but not with goat antihuman Ig. This experiment indicates the binding of [^{3}H]DNP-EACA to MOPC 315 cells is a surface phenomenon and together with the data presented above, indicates that the binding is to an Ig molecule located on the cell surface.

Association Constant of [^{3}H]DNP-EACA to MOPC 315.—The average association constant for the specific binding of [^{3}H]DNP-EACA to MOPC 315 cells was determined as previously described for [^{125}I]DNP-BSA, except that

BSA was omitted from the medium. It is difficult to wash out nonspecifically bound DNP-EACA without significantly decreasing the amount of ligand bound specifically. For this reason the studies were conducted with the cells washed one or two times. It was found that washing had little affect on the average association constant of the DNP-EACA remaining bound to cells, while the actual number of molecules bound was markedly decreased.

A Scatchard plot of the results of a representative experiment is shown in Fig. 10. After one wash the K_o's calculated for five independent experiments were 1.6, 2.1, 2.3, 4.1, and 4.25 $\times$ 10^5 L/M, an average of 2.8 $\times$ 10^5 L/M. The average number of molecules bound per cell was 1.1 $\times$ 10^7. After two wash cycles the average number of molecules bound per cell decreased approximately

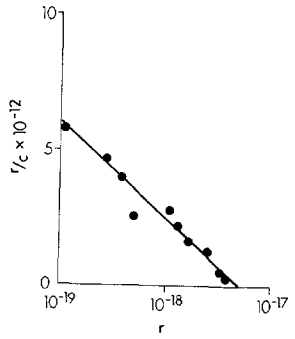

FIG. 10. Scatchard plot of binding of [^{3}H]DNP-EACA to MOPC 315 cells. r is moles of conjugate bound per MOPC 315 cell, and c is the free conjugate concentration.

4-fold, whereas the calculated K_o was unchanged. Thus, in two separate experiments, the K_o's after two washes were 2.7 and 2.8 $\times$ 10^5 L/M, and the average number of molecules bound per cell was 2.9 $\times$ 10^6. The results of these experiments indicate that the binding affinity of MOPC 315 cells for univalent DNP conjugates is approximately 300 times less than for the multivalent ligand by direct measurement of the average association constants.

The value calculated for the association constant of the reaction of cell surface Ig with [^{3}H]DNP-EACA is considerably lower than that determined for the reaction between the Ig secreted by these cells and this ligand. Equilibrium dialysis studies carried out by Dr. Young Tai Kim (Department of Medicine, Cornell University Medical College) on the binding of [^{3}H]DNP-EACA to Ig secreted by the MOPC 315 line used in these experiments yielded a value of 1.6 $\times$ 10^7 L/M for the association constant and a Sip's heterogeneity index of 1.03. These values are in agreement with those previously reported for the Ig produced by this tumor (5). The reason for the difference in the association constants determined for the free as compared with the surface Ig is not known. If it is assumed that the surface Ig molecule has binding properties identical

with those of the free Ig secreted by that cell, then the above data imply that there is an overestimate in the number of binding sites per cell when the measurements are carried out using the univalent ligand. Such an error in binding site concentration is not unlikely under conditions where low affinity interactions which are difficult to distinguish from nonspecific binding are being investigated.

Displacement of Univalent and Multivalent DNP Ligands Bound to MOPC 315.—In order to explore further the binding affinity of cell surface receptors for a multivalent as compared to a univalent ligand, the displacement rate of univalent and multivalent DNP conjugates bound to MOPC 315 was studied. In these experiments similar numbers of MOPC 315 or control cells were incubated with the univalent ligand [^{3}H]DNP-EACA or with the multivalent ligand [^{125}I]DNP-BSA at concentrations equimolar with respect to DNP groups. After allowing sufficient time for maximum binding to occur, unlabeled univalent ligand (DNP-EACA) was added in sufficient amount so as to achieve a 100-fold molar excess with respect to DNP groups. The quantity of radioactive conjugate remaining bound to cells after various time intervals was determined.

As can be seen in Fig. 11, within 4 min a 100-fold molar excess of unlabeled univalent ligand (DNP-EACA) completely displaced labeled DNP-EACA bound specifically to MOPC 315 cells, but displaced only 28% of specifically bound DNP-BSA conjugate. The relative avidity of the cell surface receptors for DNP conjugates can be evaluated from the molar concentration of DNP-EACA required to displace 50% of the bound DNP conjugate. It was found that a 5-fold molar excess of unlabeled DNP-EACA was sufficient to displace 50% of specifically bound [^{3}H]DNP-EACA within several minutes, whereas a 500-fold excess was required to displace 50% of the specifically bound [^{125}I]-

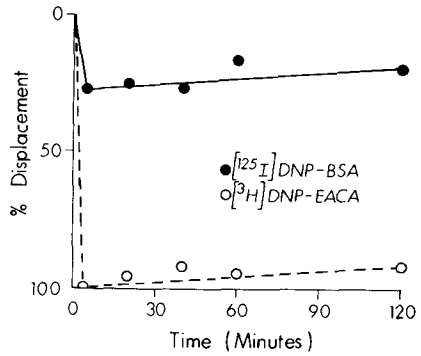

FIG. 11. Displacement of [^{125}I]DNP-BSA and [^{3}H]DNP-EACA from MOPC 315 cells by excess unlabeled univalent ligand. Similar numbers of MOPC 315 or control cells were incubated with [^{125}I]DNP-BSA (4×10^{-9} M) or [^{3}H]DNP-EACA (8×10^{-8} M) for 15 min at 27°C, and unlabeled DNP-EACA added to a final concentration of 8×10^{-6} M. The quantity of labeled conjugate remaining specifically bound to the washed cell pellet was determined at various times thereafter.

DNP-BSA. Based upon such determinations, the avidity of cell surface Ig for [^{125}I]DNP-BSA is approximately 100 times greater than for [^{3}H]DNP-EACA.

DISCUSSION

The main findings of this study are (*a*) DNP conjugates are bound specifically by cells of the murine myeloma MOPC 315; and (*b*) the avidity of cell surface Ig for multivalent conjugates is significantly greater than for the univalent compound as shown by direct measurements of association constants.

Our experiments indicate that under identical conditions, MOPC 315 cells bound up to eight times more [^{125}I]DNP-BSA than did similar numbers of control murine myeloma cells (P_3K, LPC 1, HP 76). Binding occurred by way of the DNP groups interacting with cell surface Ig. This was established by the specific inhibition of DNP-BSA binding by (*a*) DNP-EACA but not BSA, and (*b*) goat antimouse Ig but not goat antihuman Ig. Hence, the interaction of DNP conjugates to MOPC 315 cells can be regarded as analogous to the binding of specific antigen to normal antibody-secreting plasma cells.

Preliminary studies indicated that MOPC 315 cells bound a univalent conjugate with lower affinity than a multivalent one. Thus, inclusion of BSA in the medium, or extensive washing, completely inhibited specific binding of DNP-EACA but not specific binding of DNP-BSA. Consequently, it proved necessary to establish different experimental conditions for optimal specific binding of univalent and of multivalent compounds. When such conditions were employed, it could be demonstrated directly by measurement of binding affinities, and indirectly by inhibition studies, that the binding affinity of MOPC 315 cells for a multivalent conjugate was 100–300 times greater than for a univalent ligand.

The augmentation in binding of multivalent as compared with univalent ligands is of similar magnitude to that described by Davie and Paul (9). Their approach was to measure the binding of radiolabeled DNP conjugates to lymph node cells of immunized guinea pigs. Although the binding of univalent hapten could not be directly detected by this procedure, they found that increasing the degree of substitution of DNP-BSA from 4 to 19 increased by approximately 100-fold the molar concentration of DNP-lysine required for 50% inhibition of binding. The association constant which we have measured for the binding of DNP-BSA to cells is lower than that reported by Davie and Paul, a difference which presumably reflects the low binding affinity of MOPC 315 protein compared with that of induced antibody (10).

Theoretical considerations (11) and in vitro experiments (12–16) indicate that there is a marked enhancement of binding as a result of the formation of multiple bonds between individual antigen and antibody molecules. The greater binding avidity of cells for multivalent as compared with univalent ligands suggests that more than one bond is formed between the protein conjugate and cell surface antibody. While it is not possible from our results to determine the actual number of bonds formed, the magnitude of the enhance-

ment is of the same order as that reported for univalent as contrasted with bivalent interactions of antibody with antigen (16, 17).

The enhanced binding of multivalent conjugate with cell surface Ig demonstrated in this study has important biological implications. We previously postulated that a dynamic equilibrium between circulating antibody and antigen in an immunogen-containing compartment regulates the quantity of immunogen available to stimulate immunocompetent cells (3). This concept must now be extended to include the interaction of antigen with cell surface receptor sites.

In this context it should be emphasized that the binding of multivalent antigen to cells may be essentially irreversible under physiological conditions. The dissociation of a complex held by multiple independent bonds requires the simultaneous rupture of all bonds. The probability of the simultaneous rupture of multiple independent bonds is equal to the product of the probabilities of the dissociation of the individual bonds. Consequently, the dissociation of a ligand bound by multiple bonds is much less likely than the dissociation of a ligand held by a single bond, and may in fact be so slow as to render the reaction irreversible in practical terms. This stabilization of cell-bond antigen through multiple bond formation may have important consequences for the process of cell stimulation and selection by antigen.

Multivalent antigen would be particularly well suited (and may even be necessary) to stimulate immune cells. There is increasing evidence that prolonged contact of antigen with cells, measured in terms of hours to days, is required for irreversible stimulation (18–21). Formation of multiple bonds between the antigen molecule and cell surface antibody will stabilize the complex by slowing the dissociation rate and may be especially important in the stimulation of low affinity antibody forming cells. Hence, the immunogenicity of a compound should increase with increased valence. Several studies suggest that this is so (22–24), within certain limits (25). Furthermore, antigens composed of repeating antigenic units should be especially capable of forming stable complexes with immunocompetent cells. It is of interest that compounds such as bacterial polysaccharides (26, 27), polyvinylpyrrolidone (26, 28), and polymerized flagellin (29) can stimulate B cells directly.

Cell surfaces hold specific receptor sites in multivalent units so that the effective valence of the cell surface is greater than that of circulating antibody. Though the intrinsic association constant of each individual cell-associated antibody binding site may be identical with that of circulating antibody (30, 31), the cells, by being able to form multivalent interactions with antigen, have an advantage over circulating antibody in binding antigen molecules and retaining them in the bound state. These considerations may explain in part the ability of immune cells to bind, retain, and be stimulated by antigen despite the unfavorable restrictions imposed by the limited number of cells and the presence of large amounts of circulating antibody shortly after immunization.

Aggregation of antigen into polyvalent complexes may also account for the

augmentation of antibody synthesis (32–36) and for the in vitro induction of tolerance (37) by antibody. In both instances, the presence of small amounts of antibody could lead to the formation of complexes with a valence higher than that of the original antigen molecule. Thus, an appropriate quantity of antibody could increase the proportion of antigen bound to immunocompetent cells and render the binding effectively irreversible.

SUMMARY

The binding of antigen to cells with antibody on their surface has been studied in a model system consisting of murine myeloma cells (MOPC 315) and DNP conjugates. Specific binding occurred between the DNP groups of DNP conjugates and cell surface immunoglobulin.

Using this model, the binding affinities of multivalent and univalent DNP conjugates were measured directly by equilibrium-binding techniques and indirectly by displacement of bound conjugate with univalent hapten. With both approaches the multivalent conjugate was shown to bind to cells with an avidity 100–300 fold greater than the univalent hapten. Nonspecific binding of unrelated protein and repeated washing of cells was found to markedly dedecrease the specific binding of univalent conjugates, presumably because the relatively weak bonds dissociate readily.

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