Recent evidence suggests that the heterogeneous antibody population produced in response to stimulation by an antigenic determinant represents the antibody product of numerous clones of cells, each cell and cell clone producing homogeneous antibody (1–6). Thus, the specificity of the immune response derives from selective stimulation of those cells, among an individual’s entire precursor cell repertoire, whose antibody product reacts best with the stimulating antigenic determinant. This population would be unique for a given determinant, though elements of it may be stimulated by similar determinants. Inherent in the concept of unipotential cells is the ability of antigen to selectively stimulate such cells by interacting with a surface receptor whose chemical and specificity characteristics mimic those of its potential antibody product (7). Evidence exists for both the immunoglobulin nature of receptors (8–11) and their antibody-like specificity (10–13).

Analyses of the parameters of antigenic stimulation of secondary immune responses, intended to verify the above hypothesis, have indicated that, in addition to accounting for the specificity of stimulation, the mechanism of antigenic stimulation must also accommodate contributions of cell-to-cell interactions and the multiple valence of antigen-cell interactions. The role of cell-to-cell collaboration is exemplified by the amplification of the secondary response to a haptenic determinant which results from the presence of carrier-specific, thymus-dependent cells (T-cells) as well as primed bone marrow-derived precursors of anti-hapten antibody-forming cells (B-cells) (14–17). A role for multivalent interactions between antigen and cell receptors can be inferred from the lack of a correlation between antibody affinity for a determinant and the antigen concentration required to stimulate its production (18) as well as the relative inefficiency with which free hapten inhibits secondary stimulation (10, 13, 18, 19).

This study utilizes the technique of cell transfer to carrier-primed, lethally irradiated recipient mice to extend the analysis of in vitro antigenic stimulation to that...
of primary clones of anti-hapten antibody-producing cells. A comparison of the parameters of stimulation of cells from nonimmune mice with cells from immune mice reveals several qualitative differences. First, the stimulation of primary precursor cells exhibits a greater dose dependency than is seen for secondary stimulation. This is evidenced not only by the increased affinity of antibody from clones stimulated at relatively low antigen concentrations, but also by the existence of an apparent threshold affinity consonant with stimulation. Thus, while studies of antigen binding by immunocompetent cell populations show an increase in antigen-binding cells as the antigen concentration increases (11), increasing the stimulatory antigenic determinant concentration above $5 \times 10^{-7}$ M stimulates neither more cells nor cells capable of producing antibody of lower affinity. A second distinguishing characteristic of the stimulation of normal clonal precursors is the relative sensitivity of the stimulation of the majority of these cells to hapten inhibition. Finally, the stimulation of cells from unprimed mice shows a more stringent requirement for carrier recognition than cells from immunized mice which can be stimulated in the absence of carrier recognition, particularly at low antigen concentrations.

These results, together with those of earlier studies, are interpreted as indicating that the major requisites for antigenic stimulation are the ability of antigen not only to bind to receptors, but also to cause the cross-linking of receptor molecules on a cell's surface. The difference of primary and secondary cells in this interaction is interpreted as indicating that receptors of primary cells interact with antigen as if they were monovalent with respect to antigen binding while those of secondary cells appear to be at least bivalent.

**Materials and Methods**

**Antigens.**—The preparation of the protein antigens *Limulus polyphemus* hemocyanin (Hy), human serum albumin (HSA), bovine serum albumin (BSA), and bovine γ-globulin (B3'G) as well as their coupling to the 2,4-dinitrophenyl (DNP) haptenic group has been previously described (18). Analyses of the hapten-coupled immunogens showed that DNP-Hy contained 10 moles of DNP/100,000 g of Hy, DNP-HSA contained 22 moles of DNP/70,000 g of HSA, DNP-BSA contained 16 moles of DNP/70,000 g of BSA, and DNP-B3'G contained 21 moles of DNP/150,000 g of B3'G.

**Radioimmunoassay.**—The radioimmunoassay for mouse anti-DNP antibody was carried out as previously described (18, 20). 30 μg of the immunoadsorbent DNP-lysyl-bromoacetyl-cellulose was added to 0.1 ml of culture fluid or diluted serum antibody and bound antibody was detected by the addition of $^{125}$I-labeled rabbit anti-mouse immunoglobulin Fab fragment, labeled so as to detect with accuracy quantities of mouse antibody as small as 1 ng.

**Equilibrium Dialysis.**—Equilibrium dialysis was carried out on sera and culture fluids which were twice precipitated with 48% saturated ammonium sulfate and dialyzed against 0.02 M sodium phosphate and 0.15 M NaCl, pH 7.2 (PBS), as previously described (3).

**Isoelectric Focusing.**—The method used for isoelectric focusing is a modification of the method of Trump and Singer (21) suggested by Dr. Anne Good (22). Culture fluids were twice precipitated with 48% saturated ammonium sulfate and dialyzed against 0.001 M NaCl. Samples were brought to 1.6 ml in deionized 5 M urea. 0.4 ml of ampholine (pH 5-8; LKB Instruments, Inc., Rockville, Md.) and acrylamide were added so as to give a final acrylamide concentration of 5%. Focusing was carried out at 4°C for 16 hr at 350 v. Gels were frozen on dry ice, sliced, and each 2 mm slice eluted in 0.5 ml of H2O. The pH of each eluate was determined, and 0.1 ml of eluate was assayed for anti-DNP antibody activity by the radio-immunoassay.
Mouse Immunizations.—Immunization of 8–10 wk-old BALB/c mice with either DNP-Hy, Hy, HSA, or ByG was carried out by a single intraperitoneal injection of 0.1 mg of antigen in complete Freund’s adjuvant. Specific immune suppression was carried out by the intraperitoneal injection of 10 mg of DNP-HSA which had been collected as a monomer peak from Sephadex G-100 in PBS.

Spleens of immunized donor mice were taken 4–8 months after immunization when serum antibody was less than 20 ng/ml. Spleens of suppressed mice were taken 5 days after antigen injection. Carrier-immunized recipient mice were used 1–2 months after immunization.

Secondary serum antibody was obtained after a second intraperitoneal injection of 50 µg of DNP-Hy in saline. Hy-primed mice were immunized against DNP by the intraperitoneal injection of 0.1 mg of DNP-Hy in complete Freund’s adjuvant.

Cell Transfers.—Cell suspensions prepared in Dulbecco’s modified Eagle’s medium (Grand Island Biological Co., Grand Island, N.Y.) by a Teflon pestle tissue homogenizer were injected into recipient mice 6 hr after they had received 1000 R total body irradiation from a cesium source.

Fragment Cultures.—Fragment cultures of spleens of recipient mice were prepared 12–16 hr after cell transfer. Spleens were removed and sliced into 1-mm cubes by a McIlwain Tissue Chopper (Brinkmann Instruments, Inc., Westbury, N.Y.). Fragments were placed in a Petri dish containing culture medium consisting of Dulbecco’s modified Eagle’s medium supplemented with 100 µl of agamma horse serum (North American Biologicals, Inc., Rockville, Md.), 50 µl of 10-day chick embryo extract, 100 units of penicillin, 100 µg of streptomycin, and 100 units of nystatin/ml. After 6 hr, fragments were transferred individually to wells of culture plates (Linbro Chemical Co. Inc., New Haven, Conn.) in 0.25 ml of culture fluid containing antigen. Hapten inhibition was carried out by the addition of 2,4-DNP-lysine to the culture fluid 2 hr before the addition of antigen. After 3 days antigen was removed and fresh culture fluid added. Culture fluid was removed and fresh culture fluid added at 3-day intervals.

RESULTS

Table I summarizes the data obtained from analysis of culture fluids from fragment cultures of nonprimed and primed irradiated recipient mice. Donor cells were obtained from unimmunized mice as well as immunized mice and mice which had received a suppressive dose of soluble antigen 5 days previously. The results show that the number of foci producing a detectable amount of antibody increased at least sixfold when carrier-primed recipient mice were used instead of normal recipient mice. In addition, the amount of antibody released per focus was markedly increased. This enhanced in vitro stimulation was totally dependent on the use of the homologous hapten carrier complex used for immunizing the recipient. Further analysis indicated that this amplification of the response was maximal during the first 2 months after immunization of the recipient and decreased slowly thereafter. The amount of enhancement also decreased with time after irradiation of the recipient so that antigen was usually added to culture fluids within 36 hr of irradiation of the recipient.

While detectable foci could be obtained with secondary cells in fragments from normal irradiated recipients, even with heterologous carriers, the development of foci from normal donors was totally dependent on stimulation with hapten on the carrier used to immunize the recipient. At an antigen concentra-
tion presenting $10^{-7}$ M DNP, 2.9 foci were detectable per $10^6$ injected cells from the spleen of a nonimmune mouse, approximately 28% the number obtained using cells from an immunized donor. These foci generally released less antibody than secondary foci. The number of detectable primary foci could be reduced to 0.6 per $10^6$ injected cells by the injection of 10 mg of monomer DNP-HSA 5 days before harvesting spleen cells. This suppression affected only the

| Source of donor cells | Recipient primed with | Stimulating antigen ($10^{-7}$ M DNP determinant concentration) | No. fragments analyzed | Foci/10^6 injected cells producing: | Total |
|-----------------------|-----------------------|---------------------------------------------------------------|-----------------------|---------------------------------|-------|
|                       |                       |                                                               |                       | 0.7-3 ng of antibody/day | 3-6 ng of antibody/day | >6 ng of antibody/day |       |
| No cells              | Hy                    | DNP-Hy                                                         | 1140                  | 0                               | 0                          | 0              |       |
| Normal mouse          | --                    | DNP-Hy                                                         | 687                   | 0                               | 0                          | 0              |       |
| Normal mouse          | Hy                    | DNP-Hy                                                         | 6782                  | 1.6                             | 0.9                        | 0.4            | 2.9   |
| Normal mouse          | Hy                    | DNP-BSA                                                        | 386                   | 0                               | 0                          | 0              |       |
| DNP-HSA suppressed    | Hy                    | DNP-Hy                                                         | 765                   | 0.3                             | 0.2                        | 0.1            | 0.6   |
| DNP-Hy immunized      | --                    | DNP-Hy                                                         | 34,622                | 0.73                            | 0.44                       | 0.3            | 1.47  |
| mouse                 |                       | DNP-BSA                                                        | 2336                  | 0.52                            | 0.11                       | 0.03           | 0.66  |
| DNP-Hy immunized      | Hy                    | DNP-Hy                                                         | 4248                  | 2.8                             | 4.2                        | 3.6            | 10.6  |
| mouse                 | HSA                   | DNP-HSA                                                        | 978                   | 2.6                             | 3.4                        | 2.9            | 8.9   |
| DNP-Hy immunized      | ByG                   | DNP-ByG                                                        | 348                   | 3.2                             | 2.9                        | 2.6            | 8.7   |
| mouse                 | --                    | DNP-HSA                                                        | 1022                  | 0.6                             | 0.2                        | 0.2            | 1.0   |
| DNP-HSA immunized     | HSA                   | DNP-HSA                                                        | 488                   | 2.6                             | 2.4                        | 1.8            | 6.8   |
| mouse                 | HY                    | DNP-Hy                                                         | 328                   | 2.8                             | 2.8                        | 2.2            | 7.8   |

number of detectable foci; the amount of antibody produced per focus was nearly the same as normal primary foci.

Foci derived from both primary and secondary cells released maximum amounts of antibody by the 12th day after stimulation. Most foci continued to release antibody for 1-3 wk thereafter. While secondary foci generally released more antibody than primary foci, the maximum rate of antibody release per focus varied greatly both for primary and secondary foci. In cell doses between $2 \times 10^6$ and $4 \times 10^6$ cells the number of detectable primary and secondary foci
developed in carrier-primed recipients was directly related to the number of injected cells.

Fig. 1 shows the isoelectric focusing pattern obtained from ammonium sulfate precipitates of culture fluids from two primary and two secondary foci and the pattern derived from serum antibody. The patterns observed for all monofocal antibodies was markedly restricted relative to that of the serum antibody. It should be noted that while secondary monofocal antibody invariably yielded such patterns, several primary monofocal antibodies showed no antibody peak at all. This may possibly have been the result of the presence of antibodies of the IgM class which could probably not enter the acrylamide gels (23). Controls of these focusing patterns included demonstrating that eluted fractions of both monofocal and serum antibodies reelectrophoresed at the same isoelectric point.

Table II shows the data obtained by equilibrium dialysis of several primary and secondary monofocal antibodies obtained in splenic fragments from carrier-primed recipients stimulated with a DNP determinant concentration of $10^{-7}$ M. The $\alpha$ value for the heterogeneity index is presented where the data permitted
an accurate determination and in all such cases reflected homogeneity of the binding of hapten by monofocal antibody. Five of the eight values for the association constant of primary monofocal antibodies were obtained by extrapolation of the binding data to half saturation, since the small quantities of available antibody and their relatively low association constants precluded accurate measurements of binding at high hapten concentrations. However, since antibody concentrations were determined independently by the radioimmunoassay and since the hapten binding, where measurable, appeared homogeneous, the extrapolated values are probably accurate. The data for both primary and secondary monofocal antibodies showed a wide range of association constants for hapten and no correlation could be drawn from the amount of antibody released by a focus and the affinity of that antibody.

Table III summarizes the hapten-binding data obtained from an analysis of these eight primary and seven secondary monofocal antibodies developed in fragment cultures of carrier-primed recipient spleens as well as 11 secondary monofocal antibodies developed in fragments from normal spleens. The results show that the averaged association constants of 8 primary monofocal antibodies was $2.8 \times 10^6$ liters/mole which is only fivefold lower than the averaged association constants of $14.3 \times 10^6$ liters/mole obtained for secondary monofocal antibodies developed in carrier-primed recipient spleens. This latter value is almost as high as the affinity of secondary serum antibody and is higher than the averaged association constant of antibody from secondary foci developed in splenic fragments from normal recipients.

### Table II

| Sample No. | Type   | $K_a$ 7°C | $\alpha$ |
|------------|--------|----------|----------|
| 3427       | Primary| $1.8 \times 10^7$ | ---      |
| 3513       | "      | $1.0 \times 10^7$ | 0.96     |
| 2342       | "      | $6.8 \times 10^6$ | 0.98     |
| 2357       | "      | $4.1 \times 10^6$ | ---      |
| 3413       | "      | $5.2 \times 10^6$ | ---      |
| 2349       | "      | $1.1 \times 10^6$ | 0.96     |
| 2669       | "      | $6.4 \times 10^5$ | ---      |
| 2921       | "      | $2.7 \times 10^5$ | ---      |
| 2336       | Secondary| $5.7 \times 10^7$ | 0.98     |
| 2026       | "      | $3.9 \times 10^7$ | 0.98     |
| 2211       | "      | $1.8 \times 10^7$ | 0.96     |
| 2225       | "      | $1.4 \times 10^7$ | 0.97     |
| 2039       | "      | $7.8 \times 10^6$ | 0.95     |
| 2282       | "      | $6.8 \times 10^6$ | 0.98     |
| 2321       | "      | $4.2 \times 10^6$ | 0.97     |

* All foci developed in splenic fragments from Hy-primed recipient mice.
Table IV shows the number of detectable primary and secondary foci in spleen fragments of carrier-primed recipients stimulated at various antigen concentrations. The largest number of both primary and secondary foci was stimulated by a DNP determinant concentration of $10^{-8} - 10^{-7}$ M, the maximum number of primary foci being 38% that of secondary foci. Increasing the antigen concentration to $10^{-5}$ M stimulated fewer foci, but the marked suppression at that antigen concentration of the response of secondary foci developed in normal splenic fragments (2) was not observed when carrier-primed recipients were used. Some primary and secondary foci were stimulated at determinant concentrations as low as $10^{-13}$ M; however, a greater percentage of secondary than primary foci was stimulated at lower antigen concentrations. Primary and second-

**Table III**

*Comparative Binding Properties of Primary and Secondary Monofocal Antibodies*

| Sample                                      | No. of monofocal antibodies analyzed | $K_a$ (geometric mean) $\times 10^6$ liters/mole |
|---------------------------------------------|--------------------------------------|-----------------------------------------------|
| Primary foci*                               | 8                                    | $2.8 \pm 4.4$                                |
| Secondary foci*                             | 7                                    | $14.3 \pm 2.6$                               |
| Secondary foci (developed in splenic fragments from normal mice) | 11                                   | $7.7 \pm 5.8$                               |
| Primary serum antibody 12 days after immunization of carrier primed mice (pool of 6 mice) |                                      | $2.2$                                         |
| Secondary serum antibody (pool of 6 mice)   |                                      | $17.6$                                        |

* Foci developed in splenic fragments from Hy-primed recipient mice.

ary foci produced more antibody when higher antigen concentrations were used for stimulation.

Table V presents equilibrium dialysis data obtained from pooled culture fluids of several monofocal antibodies stimulated at various antigen concentrations. The affinity of the secondary foci developed in splenic fragments of carrier-primed recipients did not vary greatly when different antigen concentrations were used for stimulation. The affinity of primary foci was dependent on antigen concentration; however, the lowest affinity, $1.8 \times 10^6$ liters/mole, was achieved at $5 \times 10^{-7}$ M DNP determinant concentration, and increasing that concentration 20-fold did not lower the average affinity of antibody from stimulated foci.

Table VI presents the data obtained from the inhibition of antigenic stimulation by adding antigen in the presence of the free hapten DNP-lysine. While the stimulation of 80% of focal precursors from immune mice was inhibitable only with a large molar excess of DNP-lysine, over 80% of foci derived from non-
## TABLE IV

### The Dependence of the Focus Response on Antigen Concentration*

| Source of donor cells | Antigen determinant | Foci/10⁶ injected cells |
|-----------------------|--------------------|------------------------|
|                       | concentration (DNP on DNP-Hy) | 0.7-3 ng of antibody/day | 3-6 ng of antibody/day | >6 ng of antibody/day | Total |
|                       | moles/liter         |                        |                        |                        |       |
| Normal mouse          | 10⁻⁶               | 1.2                    | 0.9                    | 0.2                    | 2.3   |
|                       | 10⁻⁶               | 1.8                    | 1.4                    | 0.6                    | 3.8   |
|                       | 5 × 10⁻⁷           | 2.1                    | 1.3                    | 0.7                    | 4.1   |
|                       | 10⁻⁷               | 1.6                    | 0.9                    | 0.4                    | 2.9   |
|                       | 10⁻⁸               | 1.4                    | 0.8                    | 0.2                    | 2.4   |
|                       | 10⁻⁹               | 1.0                    | 0.8                    | 0.1                    | 1.9   |
|                       | 10⁻¹¹              | 0.9                    | 0.4                    | 0                      | 1.3   |
|                       | 10⁻¹³              | 0.6                    | 0.2                    | 0                      | 0.8   |
|                       | 10⁻¹⁵              | 0.2                    | 0                      | 0                      | 0.2   |

| Immunized mouse       | 10⁻⁶               | 2.8                    | 2.2                    | 1.8                    | 6.8   |
|                       | 10⁻⁶               | 3.8                    | 3.4                    | 3.0                    | 10.2  |
|                       | 5 × 10⁻⁷           | 3.2                    | 3.6                    | 3.2                    | 10.0  |
|                       | 10⁻⁷               | 2.8                    | 4.2                    | 3.6                    | 10.6  |
|                       | 10⁻⁸               | 2.7                    | 3.0                    | 2.8                    | 8.5   |
|                       | 10⁻⁹               | 2.2                    | 2.0                    | 1.8                    | 5.4   |
|                       | 10⁻¹¹              | 2.4                    | 1.8                    | 1.2                    | 5.4   |
|                       | 10⁻¹³              | 2.6                    | 1.2                    | 0.6                    | 4.4   |
|                       | 10⁻¹⁵              | 1.2                    | 0.4                    | 0                      | 1.6   |

* All foci developed in splenic fragments from Hy-primed recipient mice.

## TABLE V

### Dependence of Affinity of Pooled Monofocal Antibodies on Antigen Concentration*

| Source of donor cells | Antigen determinant concentration (DNP on DNP-Hy) | $K_a$ °C |
|-----------------------|--------------------------------------------------|---------|
|                       | (moles/liter) | $\times 10^9$ liters/mole |         |
| Normal mice           | 10⁻⁵         | 1.8†                  |         |
|                       | 10⁻⁶         | 2.0                   |         |
|                       | 5 × 10⁻⁷     | 1.8                   |         |
|                       | 10⁻⁷         | 3.0                   |         |
|                       | 10⁻⁹         | 7.2                   |         |
|                       | 10⁻¹¹        | 10.1                  |         |
| Immune mice           | 10⁻⁵         | 13.2                  |         |
|                       | 10⁻⁶         | 15.6                  |         |
|                       | 5 × 10⁻⁷     | 14.2                  |         |
|                       | 10⁻⁷         | 16.4                  |         |
|                       | 10⁻⁹         | 21.2                  |         |
|                       | 10⁻¹¹        | 22.8                  |         |

* All foci developed in splenic fragments from Hy-primed recipient mice.
† All data represent analysis of pools of at least six monofocal antibodies.
immune mice were inhibitable with hapten concentrations less than twofold
greater than the determinant concentration of the stimulating antigen.

DISCUSSION

The Clonal Origin of Primary and Secondary Monofocal Antibodies.—The
notion that single cells and the clonal progeny of a single precursor cell produce
a single immunoglobulin product has been reinforced by both studies on the
immunoglobulin product of single cells (24) and the analysis of the antibody
product of transferred cells where, statistically, a single clonal precursor was
stimulated (1–6). Recently the homogeneous antibody produced by stimulation

| TABLE VI |

*Hapten Inhibition of Primary and Secondary Monofocal Responses*

| Source of donor cells | DNP-lysine concentration | Foci/10⁶ injected cells (stimulated with DNP determinant concentration of 10⁻⁷ M on Hy) |
|-----------------------|--------------------------|----------------------------------------------------------------|
|                       | moles/liter              |                                                                   |
| Normal mouse          | 0                        | 2.9                                                               |
|                       | 10⁻⁵                     | 0.2                                                               |
|                       | 10⁻⁶                     | 0.4                                                               |
|                       | 2 × 10⁻⁷                 | 0.6                                                               |
| Immune mouse          | 0                        | 10.6                                                              |
|                       | 10⁻⁵                     | 2.2                                                               |
|                       | 10⁻⁶                     | 6.8                                                               |
|                       | 2 × 10⁻⁷                 | 8.2                                                               |

of a variety of animal species with several “restricted” antigens has also been
interpreted as the antibody product of a single stimulated clone (21, 25–27).

The results reported here extend the analysis of stimulation of clonal precur-
sors in transferred cell populations to both primary and secondary precursor
cells stimulated in fragment cultures of splenic fragments of carrier-primed
recipient mice. The single-cell origin of these monofocal antibodies was attested
to by the linear dependence of the number of detectable foci on the number of
injected cells. Additional support for this comes from the fact that, upon trans-
fer to normal recipients, secondary foci could be stimulated by the hapten on
nonhomologous carriers and thus in the absence of cells recognizing the carrier (2).

As in previous reports of secondary monofocal antibodies developed in splenic
fragments of normal recipients, both primary and secondary monofocal anti-
bodies developed in carrier-primed recipient splenic fragments were relatively
homogeneous. This homogeneity was demonstrated both by linear binding
characteristics of the antibody for hapten and restricted electrophoretic dispers-
sity of the antibody as demonstrated by isoelectric focusing. Thus, when carrier
recognition was maximized and the limiting cell for focus formation was the
antibody-forming precursor cell, monofocal antibody was homogeneous. This is in contradistinction to studies using erythrocyte antigens, where specific B-cells were probably in excess and T-cells were limiting so that monofocal antibody may have been derived from several B-cells, thereby showing heterogeneity (28, 29).

The Nature of Carrier-Specific Amplification of Stimulation.—Irradiated, carrier-primed recipient animals and cells from such animals have been used previously to enhance both primary and secondary responses (14, 30-35). The experiments reported here utilized the transfer of cells to carrier-primed, irradiated recipient mice to permit an analysis of both primary and secondary antigenic stimulation of clonal precursor cells specific for a haptenic determinant. The mechanism by which carrier priming of recipient mice served to enhance the response of clonal precursors in the donor cell population cannot be determined by the studies presented here. Since such a function has been demonstrated for carrier-primed T-cells, and this function of T-cells has been shown to be radioresistant, it would seem likely that such cells were responsible for the enhanced stimulation in carrier-primed, irradiated recipients (14, 31, 35); however, it is not yet possible to eliminate carrier-specific antibody or carrier-specific B-cells as the enhancing factor in recipient spleens.

Stimulation of precursors from nonimmune donors was possible only with the use of carrier-primed recipients, a finding similar to other reports of carrier-specific enhancement of in vitro primary responses (31, 36). The obligatory dependence of primary precursor cells on carrier recognition distinguished this cell population from cells from immune donors, some of which were stimulatable in splenic fragments from nonprimed irradiated recipients. The stimulation of secondary precursors in primed recipients differed significantly from their stimulation in splenic fragments from nonprimed recipients. Both the number of detectable foci and the amount of antibody released per focus were markedly increased when secondary precursors were stimulated in splenic fragments from carrier-primed recipients. In addition the marked inhibition of focus stimulation at high antigen concentrations was reduced when carrier-primed recipients were used. Since the averaged affinity of secondary monofocal antibodies stimulated in fragment cultures from carrier-primed recipients was higher than those detected in fragments from spleens of nonprimed mice, it seems that the maximization of carrier recognition increased the probability of stimulation at high antigen concentration, of precursors of cells whose antibody product was of high affinity. Several findings, including the fact that the average of association constants of the monofocal antibodies was similar to that of secondary serum antibody, the lack of inhibition of stimulation at high antigen concentrations, and the easy detectability of almost all secondary foci, indicate that the use of carrier-primed recipients may have allowed the expression of most, if not all, of the clonal precursors in the cultured fragments. The fact that the number of detected foci was over six times greater than that found using non-
primed recipients indicates that in previous studies of monofocal responses only a minority of the precursor cells were detectably stimulated.

*Comparative Properties of Precursor Cells from Immune and Nonimmune Donors.*—The analyses reported in this study reveal several significant qualitative differences between precursor cells obtained from immune and nonimmune donors. One difference was the aforementioned ability of secondary precursor cells to be stimulated in the absence of carrier recognition while primary precursors seem to require the presence of carrier recognition for stimulation. A second difference was the relatively greater dependence on antigen concentration of the affinity of antibody produced by stimulated precursor cells from normal donors. Perhaps the most striking difference between cells from normal and immune donors was the relatively greater sensitivity of normal precursors to inhibition of stimulation by free hapten. Only a small percentage of cells from immune donors were inhibited by concentrations of hapten less than 10-fold greater than the determinant concentration of stimulating antigen. On the other hand, less than 20% of cells from normal donors were not inhibited by such hapten concentrations. The majority of precursor cells from a secondary donor differed qualitatively from the majority of primary precursors in that most cells from a secondary donor were inhibited only at hapten concentrations 100-fold higher than the antigen determinant concentration.

The differences between the parameters of stimulation of cells from normal and immune donors is closely analogous to differences observed in the binding of hapten-protein complexes by monovalent and bivalent antibody. Bivalent anti-hapten antibody shows a relative affinity independence of binding to immunoadsorbents containing hapten-protein complexes (20) and hapten inhibition of the binding of such antibodies to these antigens, even in solution, occurs only at very high free hapten excess (37). These properties of bivalent antibody are attributable to its ability to bind both of its sites to the same antigen molecule, thus imparting a much higher affinity to the antibody-antigen complex (37–39). The binding of monovalent antibody, such as the Fab' fragment of pepsin digestion, is readily inhibited by free hapten, being almost as avid for the free hapten as for the haptenic determinant on a protein (37). In addition the avidity of monovalent antibody for polyvalent antigens directly reflects the affinity of the binding site for hapten (39). Thus, stimulation of the majority of the precursor cells from normal donors reflects the characteristics of the antigen binding exhibited by monovalent antibody while the stimulation of the majority of secondary precursor cells mimics the binding of antigen with multiple repeating determinants exhibited by bivalent 7S antibodies.

This demonstration that antigenic interactions of primary and secondary precursor cells differ qualitatively extends the implications of several other investigators who found evidence for such a difference in the relative ease of stimulation, particularly in vitro, of secondary cells (16, 19, 40). Since most
natural antigens present repeating determinants, the fact that secondary cells probably possess multivalent receptors may have considerable biological significance since the avidity of such cells for these antigens would be markedly increased over that of primary cells (41). In addition, since this antigen-induced difference in primary and secondary cells persists for at least 8 months after initial contact with antigen, and since the majority of precursor cells in an 8-wk old normal recipient are primary in nature, it seems unlikely that selective stimulation by antigen could have played a significant role in generating the population of precursor cells in a normal animal.

The Mechanism of Primary and Secondary Antigenic Stimulation.—The results reported in this study indicate that in any consideration of the mechanism of antigenic stimulation several factors must be considered. While it is clear that precursor cells specifically bind antigen (11, 13, 33, 42, 43), the act of binding antigen is not a sufficient condition for stimulation of these cells. This is best exemplified by the fact that at high concentrations both free haptenic determinants and hapten on heterologous carriers bind receptors so as to inhibit stimulation, but do not stimulate antibody production. Further evidence that antigen binding to a precursor cell is not the single prerequisite for that cell’s stimulation may be inferred from recent studies of antigen binding by lymphoid cell populations (11, 42). These studies demonstrate that the number of cells which can specifically bind an antigen (a) is higher than the expected number of precursor cells specific for such antigens, (b) is not significantly reduced by tolerance induction, and (c) is markedly increased by increasing the antigen concentration. Thus, at an antigen concentration of 1 ~g/ml as many as 2% of the cells in a cell suspension from spleens of unimmunized mice will bind hemocyanin specifically. This percentage is 1000 times greater than the number of clonal precursor cells stimulated by this antigen at that concentration (N. R. Klinman, unpublished observation). Thus, the vast majority of cells which specifically bind antigen at high antigen concentrations are not stimulated by that antigen. This finding may be analogous to the finding that a large percentage of myeloma proteins bind the DNP-lysyl determinant. Since only a few of these showed affinities approaching that of anti-DNP antibody in stimulated mice, Eisen was led to postulate that only those myelomas with high affinity for DNP were truly analogous to antibody (44). Thus, while many immunoglobulins and cells with immunoglobulin receptors bind antigens with enhanced avidity as a result of multivalent interactions, only those cells whose receptors are of relatively high affinity will be stimulated by the bound antigen.

Direct evidence for such a threshold affinity for stimulation may be obtained from an analysis of the stimulation of primary clonal precursor cells. When an antigenic determinant concentration of $10^{-7}$ M was used to stimulate these cells, the average of association constants of the monofocal antibodies detected was $3 \times 10^8$ liters/mole, with the lowest affinity antibody obtained having an association constant over $10^8$ liters/mole. When higher antigen concentrations
were used to stimulate foci, only a few more foci were detectable and the average affinity of monofocal antibodies was not significantly lowered. Thus, if precursor cells were present whose antibody product had a lower affinity for DNP-lysine, they were not stimulatable even with an antigen concentration 100 times higher. It has not been possible to extend these interpretations to the stimulation of secondary cells since such cells must already have had a sufficient affinity to have been stimulated by primary immunization; however, one might predict that such cells will show much less affinity dependence of stimulation because of the apparent multivalence of their receptors.

A second requisite for the stimulation of cells by bound antigen appears to be the ability of that antigen to interlink receptors on a precursor cell surface. This may be inferred from the inability of univalent antigenic determinants to stimulate cells although they are bound to such cells with sufficient affinity to prevent stimulation by subsequently added antigen. The necessity for cross-linkage is also consistent with previous analyses of in vitro antigenic stimulation with DNP at various levels of substitution on polymers of flagellin (45).

In addition, previous analyses of the in vitro stimulation of splenic fragments derived from nonimmune irradiated recipients reconstituted with $2 \times 10^7$ spleen cells from immune donors revealed stimulation with hapten on non-homologous carriers only at relatively low concentrations of antigen while high concentrations inhibited stimulation (18). In an analogous fashion, bivalent anti-hapten antibody forms large aggregates with hapten-protein conjugates at relatively low antigen concentrations while in antigen excess only antigen-antibody dimers are observed, with the antibody occupying both of its binding sites on the same antigen molecule (37). The fact that secondary cells are stimulated only at low antigen concentrations is consistent with the interpretation that the receptors of these cells are bivalent and that stimulation occurs at antigen concentrations where cross-linkage and aggregate formation is maximized. At high antigen concentrations where bivalent antibody molecules would not be cross-linked with antigen molecules (and in the case of primary cells, which may be monovalent and thus poorly cross-linked by nonpolymeric antigens at any concentration) stimulation may require other factors such as carrier recognition to assist in favoring the interlinked, highly aggregated arrangement.

Recent studies have demonstrated that the surface immunoglobulins of immunocompetent cells are released into the medium at a constant rate (46, 47) and that the interaction of these molecules with their cell's surface, or perhaps certain conformations of these molecules on the cell surface may be stabilized by cross-linkage of these molecules with either anti-immunoglobulin antibody or with antigen (48). If such a stabilization were necessary for antigenic stimulation, then the parameters of clonal stimulation presented in this report could be understood in terms of a mechanism for antigenic stimulation. Thus, antigen binding to a cell would be stimulatory only if that antigen interlinked receptors.
in large aggregates, making polyvalent the interaction of receptors with the cell surface. Each receptor within such a complex would have to bind antigen with sufficient affinity to ensure its retention in the polyvalent antigen-cell interaction (see Fig. 2). Thus, one might envision three reversible interactions with three different association constants: (a) $K_{a-c}$, the association constant of the cell for the antigen. This would be relatively high if the antigen is bound polyvalently by several receptors. (b) $K_{a-r}$, the association constant of the receptor for the antigen. (c) $K_{r-c}$, the association constant of the receptor for the cell surface. Stimulation would be dependent on an increase in $K_{r-c}$ resulting from this interaction becoming polyvalent through cross-linking of receptors. This would be accomplished only if the antigen were bound simultaneously by several receptors and the $K_{a-r}$ of each receptor were high enough to insure a significantly lowered dissociation rate of that receptor from the antigen-cell complex. In primary cells of low affinity, while $K_{a-c}$ may be relatively high and antigen may be bound to the cell, $K_{a-r}$ would be too low to permit sufficient stabilization of the receptors with the cell to allow stimulation. When free hapten is bound with high affinity or antigen is bound to secondary cells in concentrations which do not permit cross-linking, $K_{a-r}$ may be quite high but $K_{r-c}$ would not be increased. Since most natural antigens such as infectious

Fig. 2. A schematic diagram of the interaction of an antigen expressing repeating determinants with receptors on primary and secondary precursor cells. Receptors of primary cells are represented as expressing monovalent antigen binding while receptors of secondary cells express multivalence. The interaction of antigen with precursor cells is depicted as involving three reversible binding reactions: $K_{a-c}$, the binding of antigen to the cell; $K_{a-r}$, the binding of antigen to each receptor; and $K_{r-c}$, the binding of the receptor to the cell. Large aggregates of antigen on the surface of secondary cells can result either from cross-linking of antigens by multivalent receptors or by ancillary antigen cross-linking mechanisms such as T-cell binding of antigen. Such ancillary mechanisms are required for antigen aggregate formation on primary cells whose receptors are unable to cross-link antigens. It is postulated that receptors which bind to antigen with high affinity and are interlinked within such large antigen complexes will be stabilized in their interaction with the cell surface. Such a stabilization of $K_{r-c}$ is viewed as a requirement for stimulation.
agents present repeating determinants, such a mechanism would ensure the selective stimulation of only those primary precursor cells whose antibody product is of relatively high affinity, while secondary stimulation would be affinity independent and depend only on the ability of antigen to cross-link receptors.

These studies also indicate two important roles for carrier recognition in antigenic stimulation. First, carrier recognition appears to be essential in some instances for cross-linking receptors. Thus, when antigens present determinants monovalently or when antigen concentrations are too high to permit cross-linking of receptors, recognition of other determinants on the antigen may permit the cross-linking necessary for stimulation. It should be noted that while this cross-linking function may be the role of carrier-specific T-cells, such a function could also conceivably be carried out by anti-hapten or anti-carrier antibody, other cells with such antibody bound cytophilically, T-cells specific for the haptenic determinant, or B-cells specific for either hapten or carrier. Such ancillary cross-linking seems most essential for the stimulation of primary cells with antigens which are not large polymers since the functional monovalence of the receptors of these cells would permit only very small aggregates of receptors with an antigen molecule. As previously described (18), however, dose response analyses of secondary stimulation also indicate that stimulation is maximized and inhibition minimized where such cross-linking is favored.

This cross-linking role of carrier recognition seems particularly important in permitting stimulation of primary and secondary precursors of high affinity antibody-forming cells. Thus, only in the presence of carrier recognition was the averaged affinity of secondary monofocal antibody as high as that of serum antibody. Similarly, the affinity of both primary monofocal antibody and primary serum antibody stimulated in the presence of carrier recognition appears higher than serum antibody of mice not previously immunized to the carrier (49). In this context, immunologic learning, the increase in affinity of serum antibody with time after immunization (50), may be seen as both an initial inhibition of the highest affinity precursor cells and the subsequent selective stimulation of such cells concomitant with the development of carrier recognition. It should be noted that the affinity finally achieved in mice after prolonged immunization is only sixfold higher than the average $K_a$ of the monofocal antibodies produced by precursor cells of normal donors. This finding is consistent with previous studies showing that in some instances the initial affinity of antibody of some immunoglobulins or against certain antigens reflects an affinity almost as high as the learned potential (51, 52).

In addition to the role of carrier recognition in cross-linking where cross-linking would not otherwise occur, secondary foci stimulated in the presence of carrier recognition produced much more antibody than those stimulated in its absence. While this may reflect the stimulation of a class of cells not stimulated
in the absence of carrier recognition or simply the result of more efficient antigen presentation, a possible explanation for this amplification may be found in the close-range humoral effectors now attributed to stimulated T-cells (53, 54). Thus, not only does the amount of antibody produced by foci vary greatly, but this production can be modulated by factors related to carrier recognition. Such modulation must be taken into consideration when data obtained from antibody production or numbers of plaque-forming cells are interpreted in terms of clonal precursor cells. Most in vitro and in vivo experiments have been carried out using conditions where carrier recognition plays an important role in antigenic stimulation, and thus T-cell amplification of clonal antibody production contributes significantly to the amount of antibody measured. Evidence is still lacking, however, on the nature of stimulation by natural antigens and the biological significance of T-cell amplification in these responses.

SUMMARY

Cell transfers to carrier-immunized irradiated mice have permitted an analysis of the in vitro stimulation of clonal precursors of anti-2,4-dinitrophenyl (DNP) antibody-producing cells derived from both immune and nonimmune mice. The results indicate that: (a) carrier-specific enhancement is obligatory for stimulation of primary precursor cells and increases both the size and number of detectable foci derived from secondary precursors. (b) This carrier-specific enhancement is most apparent in the stimulation of precursors of high-affinity antibody producer cells. (c) The antibody produced by primary foci, like that of secondary foci, appears homogeneous. (d) The frequency of clonal precursors in normal spleens is 38% that in spleens from mice 4–8 months after immunization, and the number of such precursors in normal spleens can be reduced fivefold by specific suppression of donor mice with soluble antigen. (e) The average of association constants of primary monofocal antibodies, like that of primary serum antibody produced in carrier-primed mice, is less than 10-fold lower than that of secondary clonal or serum antibody. (f) The affinity of primary monofocal antibodies shows a slight dependence on stimulating antigen concentration; however, a minimum threshold affinity consonant with stimulation is apparent. (g) Free hapten inhibits antigenic stimulation of primary precursor cells at a much lower concentration than is required for the inhibition of secondary precursors.

These results are interpreted as indicating that (a) primary stimulation, like secondary stimulation, results from the selective stimulation by antigen of a population of cells differing from one another in their potential antibody product but each having only a single such product; (b) the antigen receptors of primary cells interact with antigen as if they are monovalent while receptors of secondary cells evidence multivalence; (c) antigenic stimulation appears to require both a relatively high affinity of receptors for bound antigen and an interlinking of receptors through such antigen; stimulation is thus seen as resulting from a
stabilization of receptors within antigen-receptor aggregates to the cell surface;
(d) T-cells appear to serve both in cross-linking antigens and in amplifying
the size of stimulated clones.

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