Antigenic Selection of IgG Precursor Subpopulations

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The temporal increase in the antigen-binding characteristic of the IgG antibody synthesized after primary immunization is thought to result from antigen preferentially selecting those precursors of the antibody-forming cells with higher affinity receptors. We have investigated this regulatory role of antigen on the selection and expression of IgG precursor subpopulations in rabbit lymph node populations undergoing the secondary anti-bovine serum albumin (BSA) response in vitro. The data indicate that although immunologic memory increased between 1 and 4 months after immunization there was not an increase in antigen sensitivity. We also determined the avidity of the IgG antibody secreted by PFC elicited in the cell cultures. Although the avidity of antibody secreted by PFC elicited with low concentrations of antigen was 2-fold greater than when high concentrations of antigen were employed, analyses of the avidity subpopulations of IgG PFC revealed that memory potential was not restricted to high affinity-primed cells. Moreover, populations of PFC that gave differing avidity values were similar in terms of their plaque diameters and IgG secretion rates.

It is well documented that the binding capacity of antibody increases temporally (1–6). According to the antigen selection hypothesis this temporal increase in antibody affinity is a result of antigen-induced changes in the memory cell population with the emergence of cells with higher affinity receptors which can compete more effectively for antigen (7–9). Thus maturation is due to the presence of persisting antigen which causes selective pressure in a population of cells with specificity toward the antigen. In addition, there is evidence which indicates that low concentrations of immunizing antigen lead to the production of higher affinity antibody and memory cells than do large concentrations of antigen (1, 10). Moreover, the high affinity antibody synthesized during a secondary antibody response is similar to that present just before reimmunization suggesting that memory cells of high affinity are predominant (11–13). This further suggests that either low affinity memory cells have disappeared, or been reduced to a very low percentage of total population, or cannot effectively compete for antigen in the presence of high affinity antibody and memory cells in the microenvironment. The observation that the avidity of antigen-binding cells increases after immunization has been interpreted to mean that a gradual loss of low and medium avidity antigen-binding cells occurs with time while there is a persistence of high avidity cells (14). An increase in the sensitivity of memory cells to antigen in vitro has also been shown to occur with time supporting the restriction of memory potential to high affinity cells (8). There is, however, conflicting data which indicate that maturation of the immune response does not invariably lead to the emergence and dominance of high affinity precursor cells at the expense of low affinity cells. Thus the transfer of small numbers of primed cells to irradiated recipients results in the production of lower avidity antibody than does the transfer of large numbers of cells, suggesting that the affinities of memory cells are very broad (7). More recent data obtained from a system employing microfragment cultures prepared from previously immunized rabbits indicate that large concentrations of antigen can elicit the production of low affinity antibody (15). By eliminating cell crowding through the use of microfragment cultures, it is hypothesized that low affinity memory cells can compete more effectively for antigen. The detection of the synthesis of low affinity antibody in this system indicates that low affinity memory cells are not only preserved long after immunization but under proper conditions can be stimulated.

The purpose of this research was to investigate the regulatory role of antigen on the selection and expression of IgG precursor subpopulations in rabbit lymph nodes undergoing the secondary antibody response in vitro. The results indicate that while immunologic memory increased between 1 and 4 months after immunization there was not an increase in sensitivity of the primed cells to antigen. The avidity of antibody secreted by plaque-forming cells (PFC) elicited by low concentrations of antigen was, however, 2-fold greater than when high concentrations of antigen were employed. Cultures not stimulated with antigen exhibited a spontaneous response which was predominantly of high avidity antibody. Analyses of the avidity subpopulations of IgG producing cells, however, revealed that low concentrations of antigen selected high as well as low affinity IgG precursor cells. These results demonstrate that memory potential is not restricted to high affinity IgG-primed cells.

MATERIALS AND METHODS

Animals and immunization. Female New Zealand white rabbits, 7 to 10 months of age, were immunized in each hind footpad with 1 mg/ml of bovine serum albumin (BSA), (Pentex, Inc., Kankakee, Ill.) incorporated into complete Freund's adjuvant (Difco Labs, Detroit, Mich.). Rabbits were sacrificed 1 and 4 months after immunization and the popliteal lymph nodes were removed.

Preparation of cell cultures. Popliteal lymph nodes were teased apart with sterile wire screens and the resulting cell suspension was filtered through a double layer of sterile cheese cloth. The cells were washed twice in Eagle's minimum essential medium (MEM, Grand Island Biological Co., Grand Forks, N.
Island, N. Y.) and adjusted to a final concentration of $2 \times 10^7$ nucleated cells/ml in MEM supplemented with MEM vitamins, nonessential amino acids, penicillin, streptomycin, and 20% fresh heat-inactivated normal rabbit serum (NRS). Two-milliliter aliquots of the cell suspension were pipetted into 35- x 10-mm plastic Petri plates (Lux Scientific Corp., Thousand Oaks, Calif.) and challenged with various concentrations of BSA. The cultures were incubated at 37°C in 5% CO₂-air atmosphere on a rocker platform (Belco Glass Inc., Vineland, N. J.) oscillating at 8 to 10 cycles/min. Antigen was removed 24 hr later by washing the cells in each culture two times in a total volume of 20 ml of MEM containing 1% heat-inactivated NRS followed by resuspension of the cells in complete medium. The cells were centrifuged and resuspended in fresh complete culture medium 72 hr after initiation of cultures.

Preparation of sensitized sheep red blood cells. The carbodiimide method of Gunn and Roszman (16) was employed to couple BSA to sheep red blood cells (SRBC). Cells were coupled in the presence of 100 mg/ml of BSA since preliminary experiments demonstrated that this concentration yielded maximal numbers of IgG PFC. The sensitized SRBC were resuspended to a final concentration of 7% (v/v) in MEM.

Plaque assay technique. A modification of the Cunningham and Szenberg liquid plaque assay was employed (17). Two siliconized microscope slides were taped together with double side tape (Dubi-Stik, Kleen-Stik Prods., Newark, N. J.) to form two chambers with a volume of approximately 0.05 ml each. IgG plaques were developed by incubation with goat anti-rabbit IgG serum (Cappel Laboratory, Downington, Pa.) at a final concentration of 1:40. This facilitating antiserum caused complete inhibition of IgM PFC (18). The chambers were incubated at 37°C for 120 min to develop the IgG plaques. After incubation the plaques were counted under 35 × magnification. Measurement of plaque diameters were accomplished employing a microscope with a calibrated eyepiece under 100 × magnification. A total of 100 plaques were measured for each individual experiment and the results were expressed as a percentage. In order to determine the secretion rate of IgG antibody by PFC and PFC/10⁶ nucleated cells were filled with aliquots of cell suspension containing from 200 to 300 PFC/ml and the slides were placed in a 37°C incubator. Thirty minutes after incubation and at 15-min intervals thereafter slides were removed and counted as previously described.

Plaque inhibition assay technique. The method of Anderson (19) was employed to determine the percentage of inhibition of IgG PFC by various concentrations of BSA. Aliquots of cell suspensions from cultures stimulated with antigen concentrations which yielded optimum secondary antibody responses in vitro were assayed by this method. In addition, cultures stimulated with 100 μg/ml of antigen and those receiving no antigen were assayed. That concentration of BSA which inhibited 50% of PFC was taken as the avidity ($I_o$).

Analysis of avidity subpopulations. The method of Davie and Paul (20) was employed to determine the number IgG PFC in each avidity subgroup.

RESULTS

Kinetics of the in vitro secondary IgG antibody response. Cultures of lymph node cells prepared from rabbits 1 and 4 months after primary immunization were challenged with various concentrations of antigen. The appearance of IgG PFC on various days of the response is illustrated in Table I. Maximum numbers of IgG PFC appeared on the 5th day of culture and the response was biphasic with optimum numbers of PFC elicited in cultures stimulated with 0.1 μg/ml as well as with 10 μg/ml and 100 μg/ml of BSA. Cultures which were not stimulated with antigen also exhibited a significant IgG PFC response. Similar experiments were performed with cultures prepared from rabbits primarily immunized 4 months previously (Table II). Maximum numbers of IgG PFC appeared on the 5th day of the response with a rather broad dose-response profile observed when cultures were stimulated with between 0.01 μg/ml and 10 μg/ml of BSA. An immune response was also detected in cultures not stimulated with antigen and could be reduced to about 90% of control level by subjecting the cell suspensions to procedures which remove macrophages.

Avidity of IgG antibody secreted by PFC. Inhibition experiments were performed to determine the avidity of IgG antibody secreted by PFC obtained from cultures stimulated with high and low concentrations of antigen. The data from three to five separate experiments showing inhibition of IgG PFC present at 1 month after primary immunization and obtained from cultures on the 5th day of the in vitro antibody response are

<table>
<thead>
<tr>
<th>Antigen Concentration</th>
<th>PFC/10⁶ Nucleated Cells at Day: a,b,c</th>
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</thead>
<tbody>
<tr>
<td>µg/ml</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>392 ± 233 590 ± 360 268 ± 91</td>
</tr>
<tr>
<td>0.001</td>
<td>178 ± 65 321 ± 58 208 ± 102</td>
</tr>
<tr>
<td>0.01</td>
<td>296 ± 115 545 ± 149 330 ± 103</td>
</tr>
<tr>
<td>0.1</td>
<td>626 ± 283 1244 ± 385 620 ± 146</td>
</tr>
<tr>
<td>1</td>
<td>599 ± 188 643 ± 154 538 ± 190</td>
</tr>
<tr>
<td>10</td>
<td>456 ± 170 1067 ± 269 606 ± 168</td>
</tr>
<tr>
<td>100</td>
<td>622 ± 195 1080 ± 477 564 ± 198</td>
</tr>
</tbody>
</table>

* 301 ± 51 PFC/10⁶ nucleated cells present in lymph node cell suspensions 1 month after primary immunization.
* Day after initiation of culture.
* Expressed as mean ± standard error of the mean of values obtained from 7 to 11 separate animals.

Influence of antigen concentration on the in vitro secondary antibody response of lymph node cells obtained from animals 4 months after primary immunization

<table>
<thead>
<tr>
<th>Antigen Concentration</th>
<th>PFC/10⁶ Nucleated Cells at Day: a,b,c</th>
</tr>
</thead>
<tbody>
<tr>
<td>µg/ml</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1179 ± 876 1664 ± 1258 218 ± 51</td>
</tr>
<tr>
<td>0.001</td>
<td>471 ± 219 1305 ± 1158 505 ± 158</td>
</tr>
<tr>
<td>0.01</td>
<td>938 ± 410 2712 ± 1576 506 ± 158</td>
</tr>
<tr>
<td>0.1</td>
<td>2022 ± 629 3006 ± 1420 872 ± 281</td>
</tr>
<tr>
<td>0.5</td>
<td>1890 ± 723 4057 ± 955 2852 ± 2210</td>
</tr>
<tr>
<td>1</td>
<td>1878 ± 416 3480 ± 1038 554 ± 150</td>
</tr>
<tr>
<td>10</td>
<td>1508 ± 315 3244 ± 915 338 ± 102</td>
</tr>
<tr>
<td>100</td>
<td>1628 ± 363 1778 ± 473 349 ± 121</td>
</tr>
</tbody>
</table>

* 325 ± 134 PFC/10⁶ nucleated cells present in lymph node cell suspensions 4 months after primary immunization.
* Day after initiation of culture.
* Expressed as mean ± standard error of the mean of values obtained from five to seven separate animals.
presented in Figure 1. The highest avidity IgG antibody was produced by PFC obtained from cultures which were not stimulated with antigen. The average $I_{50}$ value for this antibody was 0.46 μg BSA and it is apparent that greater numbers of PFC were inhibited by lower concentrations of BSA than were any of the other populations of PFC tested. Cultures stimulated with a 0.1 μg/ml of BSA elicited IgG antibody which had $I_{50}$ values approximately 2-fold less than antibody secreted by PFC obtained from cultures stimulated with 100 μg/ml of BSA but was very similar to the $I_{50}$ value of the antibody secreted by PFC present at 1 month after immunization. Similar studies were performed with animals immunized 4 months previously and the mean of the results of 3 or 4 separate experiments are summarized in Figure 2. Cultures which were not stimulated with antigen produced the highest avidity antibody with an average $I_{50}$ value of 0.58 μg BSA as compared to 1.5 μg BSA and 3.7 μg BSA for antibody produced by cultures stimulated with 0.5 μg/ml and 100 μg/ml of antigen, respectively. The average avidity of antibody secreted by PFC present in lymph node cell preparations 4 months after immunization was 1.9 μg BSA. In Table III is presented a summary of the $I_{50}$ values from inhibition experiments performed with lymph node cells obtained from animals primarily immunized 1 and 4 months previously.

Secretion rates of IgG antibody and plaque diameters. PFC obtained from cultures either stimulated or unstimulated with

![Figure 1](http://www.jimmunol.org/)

*Figure 1*. Inhibition of IgG anti-BSA plaque formation by BSA. PFC obtained on day 5 from cultures prepared from animals 1 month after primary immunization and challenged *in vitro* with 0.1 μg/ml BSA, • •; 100 μg/ml BSA, • •; and no antigen, • •. PFC present in lymph node cell suspensions 1 month after primary immunization, O--O.

![Figure 2](http://www.jimmunol.org/)

*Figure 2*. Inhibition of IgG anti-BSA plaque formation by BSA. PFC obtained on day 5 from cultures prepared from animals 4 months after primary immunization and challenged *in vitro* with 0.5 μg/ml BSA. • •; 100 μg/ml BSA, • •; and no antigen, • •. PFC present in lymph node cell suspensions 4 months after primary immunization, O--O.
antigen were compared in terms of the rate of secretion of IgG antibody and the diameter of the plaques. In Figure 3 is shown the plaque diameter distribution profiles of representative experiments employing PFC from cultures stimulated with either low or high concentrations of antigen or unstimulated. The distribution profiles were similar regardless of the antigen concentration used to stimulate the cultures and the length of time which had lapsed after primary immunization. The secretion of antibody by PFC obtained from these cultures was also not different, as illustrated in Figure 4.

Analysis of IgG avidity subpopulations. A more accurate reflection of the effects of antigen concentration on a population of primed cells can be obtained by enumerating the number of PFC which are inhibited by successive antigen concentration intervals (20). In Figure 5 is illustrated the mean of the results of three to five separate experiments showing the percentage of total PFC contained in various avidity subgroups in cultures prepared from animals 1 month after primary immunization.

![Graph of IgG avidity subpopulations.](image)

**Figure 5.** Mean of results from three to five separate experiments showing the percentage of total PFC contained in various avidity subgroups in cultures prepared from animals 1 month after primary immunization.

### Table III

**Comparison of the avidities of IgG antibody secreted by PFC obtained from lymph node cultures prepared from animals 1 month and 4 months after primary immunization**

<table>
<thead>
<tr>
<th>Antigen Concentration</th>
<th>Day of Culture</th>
<th>Aviditya,b</th>
</tr>
</thead>
<tbody>
<tr>
<td>µg/ml</td>
<td>1 month</td>
<td>4 months</td>
</tr>
<tr>
<td>0</td>
<td>1.68 ± 0.26</td>
<td>1.90 ± 0.10</td>
</tr>
<tr>
<td>5</td>
<td>0.46 ± 0.12</td>
<td>0.58 ± 0.07</td>
</tr>
<tr>
<td>0.1</td>
<td>1.74 ± 0.17</td>
<td>1.40 ± 0.28</td>
</tr>
<tr>
<td>0.5</td>
<td>3.60 ± 0.15</td>
<td>3.70 ± 0.25</td>
</tr>
<tr>
<td>100</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a Micrograms/milliliter of BSA required to inhibit 50% of PFC (I₅₀).
*b PFC obtained from day 5 cultures.

Of the PFC present initially in 1-month lymph node cell populations, there were approximately equal percentages found in all avidity subgroups except the lowest subgroup (8 to 32 µg BSA). Cultures not stimulated with antigen contained a majority of PFC in the two higher avidity subgroups (0 to 0.5 µg BSA and 0.5 to 2 µg BSA). Of the cultures stimulated with antigen, those that received a 0.1 µg/ml of antigen had the smallest percentage of PFC in the lowest avidity subgroup (8 to 32 µg BSA) whereas cultures stimulated with 100 µg/ml of antigen had no PFC in the highest avidity subgroup (0 to 0.5 µg BSA).
Similar avidity subgroup analyses were performed with PFC obtained from animals immunized 4 months previously and the mean of the results of four separate experiments is shown in Figure 6. A flat distribution profile was observed with PFC present in lymph node cell suspensions 4 months after immunization. In contrast, PFC elicited in cultures not stimulated with antigen were mainly producing high avidity IgG antibody as were PFC obtained from cultures stimulated with 0.5 μg/ml of antigen. Cultures stimulated with 100 μg/ml of antigen, however, contained no PFC in the highest avidity subgroup but substantial numbers in all of the other subgroups.

DISCUSSION

According to the antigen selection hypothesis the binding capacity of antibody increases with time after primary immunization and is attributed to the selection of primed cells with progressively higher affinity receptors by limiting antigen concentration (1). In the present study the regulatory role of antigen on the selection and expression of IgG-precursor cells in rabbit lymph node cell populations undergoing the secondary antibody response in vitro was investigated. An increase in immunologic memory was observed between 1 and 4 months after primary immunization as evidenced by the fact that 2 to 5 times as many PFC were elicited in cultures prepared from animals immunized 4 months versus 1 month previously. This increase in memory is due to the presence of persisting antigen in the draining lymph nodes since a spontaneous response was obtained in cultures not stimulated with antigen. If the affinity of the primed cells increased during this 3-month period, it should have been reflected in increased sensitivity of the cells to antigen. This was not found to be the case. Cultures prepared from animals immunized 4 months previously elicited increased numbers of IgG PFC to all concentrations of antigen employed when compared with similarly stimulated cultures prepared from 1-month-immunized animals. However, a marked increase in the sensitivity of these cultures to antigen was not observed. Bullock and Rittenberg (8) compared the responses of spleen cells obtained from mice 6 and 18 weeks after immunization and found the optimal numbers of IgG PFC were obtained with 100-fold less antigen at the 18-week period. Moreover, they observed that immunologic memory waned with time and concluded that their results were consistent with the interpretation that only memory cells with high sensitivity for antigen continued to be selected in vivo.

Others have observed that there is no change in the concentration of antigen required to stimulate the incorporation of 3H-thymidine into the DNA of cells obtained either early or late after immunization (21). However, there was observed a temporal increase in the affinity of the antibody. While our
results indicate there was no change in the avidity of the IgG antibody synthesized by PFC between 1 and 4 months after immunization, previous results from this laboratory have demonstrated that the avidity of IgG antibody to BSA does increase with time and that this maturation is complete within 1 month after immunization (18). Thus, the data suggest that persisting antigen is selecting a wide spectrum of memory cells with respect to affinity and that expansion of this heterogeneous population is occurring between 1 and 4 months after immunization.

Analyses of the avidity of IgG antibody secreted by PFC elicited in cultures obtained from animals immunized 1 or 4 months previously revealed the following. First, low antigen concentrations elicited the production of IgG antibody which was approximately 2-fold higher in average avidity than antibody elicited by high antigen concentration. Secondly, there was no difference between the avidities of antibody produced in 1-month versus 4-month cultures stimulated with either low or high concentrations of antigen. Finally, analyses of the IgG PFC subpopulations elicited in 1- and 4-month cultures with high and low concentrations of antigen displayed heterogeneity. However, low concentrations of antigen stimulated the appearance of substantially more high avidity PFC than did high concentration of antigen, indicating that antigen selection is operative. These results clearly indicate, however, that IgG-precursor cells of varying affinity are present in the lymph node populations and can compete for antigen and that antigen-driven maturation of the response in the presence of persisting antigen does not continue between 1 and 4 months after immunization. Using a different system Macario et al. (15) recently described similar findings indicating that memory potential with regards to affinity is not restricted after primary immunization. They employed small tissue fragments containing about $10^6$ cells prepared from lymph nodes and suggested that low cell numbers favor the expression of low affinity memory cells. The results with our system, which employs cell suspensions at maximum cell density, do not support this contention.

Cultures stimulated with 100 μg/ml of BSA had avidity subpopulations of PFC considerably different from the subpopulations in cultures stimulated with low concentrations of antigen. There was a complete absence of the highest avidity PFC subpopulation indicating that partial high zone tolerance had occurred. Others have reported that the in vitro secondary antibody response can be totally suppressed with milligram quantities of antigen as measured by the Farr method does not in every instance correlate well with values obtained by the plaque inhibition technique. The major reason given for this apparent discrepancy is the expression of low affinity cells by antigen via cytophilic antibody adherent to the macrophage surface and that this antigen can stimulate lymphocytes. They suggest, furthermore, the biologic role of this macrophage-bound cytophilic antibody is to provide a means of concentrating a sufficient amount of antigen on the macrophage surfaces to stimulate a secondary antibody response even when antigen is limiting. Our results confirm that such an event is indeed occurring under the in vitro conditions employed. Sufficient quantities of antibody are being synthesized at 1 and 4 months after immunization to provide cytophilic antibody. It should be noted that the avidity of the antibody secreted by PFC obtained from cultures undergoing a spontaneous response is 2 to 3 times greater than that observed at 1 or 4 months after immunization. This suggests that in vivo mechanisms are different from in vitro mechanisms in terms of selection and stimulation of memory cells by antigen.

Recently North and Askonas (33) observed that the affinity of monoclonal antibody as measured by the Farr method does not in every instance correlate well with values obtained by the plaque inhibition technique. The major reason given for this apparent discrepancy is the difference in plaque diameters between different clones and among plaques from a given clone. The authors conclude that the plaque inhibition assay is only of limited usefulness for the analysis of monoclonal antibody responses. The results of our study indicate that the plaque diameters as well as the secretion rates of IgG antibody are very similar regardless of the avidity of the antibody being synthesized. It is thus apparent with the system which we are employing that variations in plaque diameters and secretion rates are not occurring and that the interpretation of our plaque inhibition data is valid. Moreover, it is well documented that the relative affinity or avidity of antibody as measured by plaque inhibition correlates well with values determined for serum antibody (34, 35). A correlation, furthermore, was found to exist between the rate of increase in the avidity of IgG antibody at the serum and cellular level with the increased avidity of antigen-binding cells during the immune response to dinitrophenol-guinea pig albumin (20). Finally, mathematical considerations of plaque inhibition has confirmed the validity of the technique at the theoretical level (36, 37).
REFERENCES