COMPLETE INHIBITION OF THE EXPRESSION
OF AN IDIOTYPE
BY A MECHANISM OF B-CELL DOMINANCE*

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By using isoelectric focusing spectra or idiotypy as markers, it has been shown that clones of B cells producing identifiable antibody molecules can be adoptively transferred to syngeneic animals (1–5); cells can be obtained from ascitic fluids (6) as well as spleen. The work of Bangasser et al. suggested that, under certain circumstances, the dominance of the B-cell clones transferred could prevent the appearance of anti-hapten antibodies with the same specificity, but of a different idiotype, that would otherwise have appeared upon subsequent immunization (6). The experiments were based on the observation that all A/J mice produced antibodies to the p-azophenylarsonate group (anti-Ar\(^1\) antibodies) some of which share a cross-reactive idiotype (CRI) (7) and that the appearance of this idiotype can be suppressed by prior inoculation of rabbit anti-idiotypic antibodies (8). Suppressed mice produce normal concentrations of anti-Ar antibodies which lack the CRI (9). When spleen cells from suppressed, nonimmune mice were adoptively transferred to lethally irradiated recipients, the latter, when immunized, produced anti-Ar antibodies lacking the CRI. When the recipients received normal spleen cells 17 days after the adoptive transfer of suppressed cells the anti-Ar antibodies formed upon subsequent immunization possessed the idiotype (6). If, however, the animals were challenged with antigen on day 7, between the adoptive transfers of suppressed and normal cells, the anti-Ar antibodies formed when the animals were immunized lacked detectable amounts of the CRI. This suggested the possibility that the lymphocytes transferred from a suppressed animal, when brought into contact with antigen, generated memory B cells incapable of producing the CRI, which were selectively triggered by antigen and prevented the expression of idiotype by the normal cells that were transferred on day 17. An alternative interpretation was that the injection of antigen on day 7 very rapidly generated the production of idiotype-specific suppressor T cells, shown by Eichmann (3) to be produced, after a prolonged waiting period, by mice inoculated with anti-idiotypic antibodies.

Subsequent experiments of Ward et al.\(^2\) demonstrated the possibility of suppressing the appearance of the CRI by adoptive transfer of B cells, from idiotypically suppressed hyperimmunized A/J mice that were producing anti-Ar antibodies lacking CRI, into

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1 Abbreviations used in this paper: Ar, p-azophenylarsonate; BGG, bovine IgG; CFA, complete Freund's adjuvant; C\(_h\)-region, constant region of the heavy chain; CRI, cross-reactive idiotype; KLH, keyhole limpet hemocyanin.

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mildly irradiated (200 rads) A/J recipients. Transfers of limiting dilutions of separated populations of B and T cells indicated that, on a numerical basis, B cells were at least as effective as T cells in transferring the suppression, and that suppression could be induced by B-cell populations containing far fewer T cells, as contaminants, than were required to induce the suppressed state. Thus, both sets of experiments are consistent in suggesting that memory B cells, possessing receptors that recognize the p-azophenylarsionate group but lack the CRI, can compete effectively for antigen and prevent the expression of the idiotype in recipient animals that would otherwise have produced it.

Because it is impossible to rule out the presence of a small percentage of T cells in enriched B-cell populations, the possibility existed that the virtually complete suppression of CRI, as a consequence of B-cell dominance, might require the synergistic action of a small number of suppressor T cells. The experiments reported here were designed to test for suppression of idiotype under circumstances in which idiotype-specific suppressor T cells were not likely to be present. The protocol consisted in immunizing BALB/c mice, which are CRI-negative, against the Ar hapten group, and transferring their immune spleen cells into mildly irradiated C.AL-20 mice; the latter possess the allotype of the AL/N strain on a BALB/c genetic background and are capable of producing the CRI. This procedure resulted in essentially complete inhibition of the synthesis of the CRI upon subsequent immunization, as determined by a sensitive assay. The inhibitory cells were insensitive to treatment with anti-Thy-1.2 and complement. Other experiments were carried out to exclude a role for (hypothetical) helper T cells specific for idiotypes other than the CRI or for carrier-specific suppressor T cells. Implications with respect to the role of clonal dominance in the immune response will be discussed.

Materials and Methods

BALB/c mice were obtained from The Jackson Laboratory, Bar Harbor, Maine. The mice were 8-14 wk of age at the start of each experiment. Mice of the congenic C.AL-20 strain, which have genes controlling the Cg-region allotype of the AL/N strain on a BALB/c background, were the gift of Dr. Michael Potter, National Institutes of Health. Rabbits were obtained from the White Pine Rabbitry, East Douglas, Mass. Keyhole limpet hemocyanin (KLH) was obtained from Calbiochem, La Jolla, Calif. p-aminophenylarsionic acid, obtained from Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N. Y., was recrystallized from a mixture of ethanol and water. Bovine IgG (BGG) and bovine serum albumin (Fraction V) were obtained from Sigma Chemical Co., St. Louis, Mo. Complete and incomplete Freund's adjuvants were purchased from the Difco Laboratories, Detroit, Mich., and ascitic fluid containing anti-Thy-1.2 (AKR anti-C3H) antibodies from Litton Bionetics Co., Kensington, Md. RPMI-1640 medium and decomplemented fetal calf serum were obtained from Grand Island Biological Co., Grand Island, N. Y.

The following methods have been described: conjugation of proteins with p-aminophenylarsionic acid by diazotization (10); induction in mice of ascitic fluids containing anti-Ar antibodies, using KLH-Ar as immunogen (11); specific purification of anti-Ar antibodies, production of rabbit anti-idiotypic antibodies against the anti-Ar antibodies of A/J mice, and the radioimmunoassay for CRI, using 10 ng of 125I-labeled specifically purified anti-Ar antibodies as ligand and goat anti-rabbit Fc as the antiglobulin reagent (7); specific purification of rabbit anti-mouse IgG (12). Proteins were iodinated by the method of Hunter, by using chloramine-T (13). Anti-Ar antibodies in serum or ascitic fluids were quantitated essentially by the procedure of Klinman et al. (14), which employs polyvinyl microtiter plates as the initial adsorbent for bovine serum albumin-Ar. The wells are then exposed to unknown samples containing anti-Ar antibodies, followed by 125I-labeled, specifically purified rabbit anti-mouse IgG. A standard curve is constructed by using sera containing known concentrations of precipitating anti-Ar antibodies.
Single cell suspensions were obtained by teasing spleens through stainless steel mesh or between two frosted glass plates. To count lymphocytes, the erythrocytes were first lysed with ammonium chloride solution (15). Viability, as estimated by trypan blue exclusion, exceeded 85%. Data presented in the tables refer to numbers of viable lymphocytes. Spleen cells were transferred adoptively, by the intravenous route, into recipient mice which had received 200 rads over a period of 5 min from a $^{137}$Cs source (J. L. Shepard and Associates, Glendale, Calif.). The recipient mice were immunized by two inoculations of 0.25 mg of KLH-Ar emulsified in complete Freund's adjuvant (CFA), on days 3 and 10 after adoptive transfer, followed by two additional injections of the antigen in incomplete Freund's adjuvant on days 17 and 24. Mice were bled retro-orbitally on day 31 and their sera tested for content of anti-Ar antibodies and of CRI.

For treatment of cells with anti-Thy-l.2 reagent, the complement used was obtained by screening sera of 2-pound rabbits for low cytotoxicity. The complement-containing serum was adsorbed according to Boyse (16). Anti-Thy-l.2 plus complement killed a maximum of 55% of A/J splenic leukocytes and over 90% of leukocytes not retained on a nylon wool column. Nonspecific killing by each reagent alone was less than 10%.

Results

The results in Table I indicate, first, that irradiated C.AL-20 mice are capable of producing anti-Ar antibodies with the CRI when immunized with KLH-Ar. The adoptive transfer of $10 \times 10^6$ or $50 \times 10^6$ spleen cells from nonimmune BALB/c mice or from BALB/c mice that had been hyperimmunized with KLH (footnote, Table I) did not prevent the appearance of the idiotype upon subsequent immunization with KLH-Ar. The amounts of anti-Ar antibodies required for 50% inhibition in the radioimmunoassay for the idiotype were comparable to those in the controls. Some evidence for a reduction in the concentration of idiotype, per unit weight of anti-Ar antibodies, can be seen in the group that received $75 \times 10^6$ cells. This may be attributable to the production of anti-Ar antibodies of BALB/c origin in the mice which received this larger number of cells, which would lower the proportion of molecules with the CRI.

In contrast, the adoptive transfer of only $10 \times 10^6$ cells from BALB/c mice that had been immunized with KLH-Ar caused essentially complete suppression of the CRI in the C.AL-20 recipients. Since it seems unlikely that BALB/c mice, which are incapable of producing the idiotype (7, 17), would develop idiotype-specific suppressor T cells upon immunization with KLH-Ar, the data suggest that the suppression of idiotype was caused by the selective capture of antigen by BALB/c B cells with receptors for the $p$-azophenylarsonate group, that were present in the hyperimmunized donors. The average anti-Ar titers for groups 1 and 4 in Table I were 0.8 and 0.4 mg/ml, respectively.

Further evidence that the inhibition indicated by the data in Table I is not attributable to suppressor T cells was obtained by treating cells from the donor mice in vitro with anti-Thy-l.2 and complement before the transfer (Table II). In these experiments the cells remaining after treatment of $20 \times 10^6$ splenic lymphocytes, from BALB/c mice immunized with KLH-Ar, were transferred into each recipient; 51% of the cells were killed. The remaining cells still caused essentially complete suppression of the idiotype upon subsequent immunization of the C.AL-20 recipients.

To investigate the persistence of the suppressed state the last group of recipients in Table I, that had received $100 \times 10^6$ cells from BALB/c mice immunized with KLH-Ar, were allowed to rest for 5 mo. The nine survivors
TABLE I

*Inhibition of CRI in C.AL-20 Mice by Transfer of Spleen Cells from BALB/c Mice Immunized with KLH-Ar*

| Donor of cells transferred | No. of recipient mice | No. of cells transferred to each mouse | Nanograms anti-Ar Ab required for 50% inhibition* |
|----------------------------|-----------------------|----------------------------------------|-------------------------------------------------|
| None§                      | 19                    | None                                   | $41 \times 10^{-4}$ 44, 45, 48, 90, 93, 94, 110, 200, 280, 430, 520, 560, 570, 590, 730, 750, 800, 1,200 |
| Nonimmune BALB/c           | 5                     | 10                                     | 38, 56, 96, 140, 190                                      |
|                            | 5                     | 50                                     | 39, 45, 140, 150, 190                                      |
|                            | 5                     | 75                                     | 110, 320, 500, 500, 2,000  |
| BALB/c immunized with KLH¶ | 4                     | 10                                     | 49, 56, 86, 110                                      |
|                            | 3                     | 50                                     | 41, 150, 260                                      |
|                            | 4                     | 75                                     | 70, 130, 530, 4,800                                      |
| BALB/c immunized with KLH-Ar¶ | 5                   | 10                                     | >13,000 to >25,000                                   |
|                            | 4                     | 50                                     | >24,000 to >29,000                                   |
|                            | 10                    | 100                                    | >9,000 to >15,000                                   |

* All C.AL-20 mice received 200 rads, 4 h before the adoptive transfer. They were then immunized by four i.p. inoculations of 0.25 mg of KLH-Ar in CFA over a 24 day period and bled 1 wk later.
† 10 ng of labeled ligand were used in the radioimmunoassay.
§ C.AL-20 mice were irradiated (200 rads) and immunized (without receiving cells).
|| Each value represents an individual mouse.
¶ A single pool of donor cells was used. Donors had been immunized by 4 weekly i.p. inoculations of 0.25 mg of antigen in complete Freund’s adjuvant (volume ratio of antigen to adjuvant, 1:1). Adoptive transfers were carried out 2 wk after the last injection.

TABLE II

*Effect of Treatment with Anti-Thy-1.2 and Complement on the Capacity of Spleen Cells from BALB/c Mice Immunized with KLH-Ar to Suppress CRI in C.AL-20 Recipients*

| Treatment with anti-Thy-1.2 plus complement | Number of C.AL-20 recipients | Nanograms anti-Ar Ab required for 50% inhibition† |
|--------------------------------------------|------------------------------|-------------------------------------------------|
| (-)                                        | 7                            | >4,000 to >6,500                                 |
| (+)                                        | 5                            | >3,500 to >11,500                                |

* Each recipient mouse received 200 rads and 20 × 10⁶ splenic lymphocytes, or the cells remaining after treatment of 20 × 10⁶ lymphocytes with anti-Thy-1.2 and C; the treatment killed 51% of the cells. A single pool of 300 × 10⁶ cells was used; 100 × 10⁶ cells were treated and aliquots injected into recipient mice. The immunization procedure is described in footnote*, Table I.
† 10 ng of labeled ligand was used in the radioimmunoassay.

were injected i.p. with 0.25 mg of KLH-Ar in CFA and bled 1 wk later. Anti-Ar titers ranged from 0.3 to 1.5 mg/ml. 25 μl of serum from each of the mice caused less than 30% inhibition of binding of 10 ng of labeled ligand in the radioimmunoassay for CRI.
Two additional hypotheses involving T cells were considered that might account for the data. These hypotheses, which are considered in detail in the Discussion, involve a role for BALB/c idiotype-specific helper T cells or KLH-specific suppressor T cells, which might have been present in small numbers in the spleen cells treated with the anti-Thy-1.2 reagent and complement.

An experiment was performed in which it was believed that neither of these mechanisms would be operative. The BALB/c donors were primed with BGG-Ar, rather than KLH-Ar, and the C.AL-20 recipients were immunized with KLH-Ar after the adoptive transfer. Under these circumstances one would not expect to generate idiotype-specific helper T cells with specificity for KLH, and carrier-specific suppressor T cells should not be relevant. This would, however, stimulate B cells in the BALB/c donors with anti-Ar receptors lacking CRI. If B-cell competition is responsible for the inhibition, the cells from such donor mice would be expected to cause inhibition of the production of CRI. The data in Table III indicate that cells from such donors were highly inhibitory when 10 × 10^6 cells or more were transferred; 1 × 10^6 cells did not prevent the synthesis of the CRI.

The transfer of 10 × 10^6 or 50 × 10^6 lymphocytes from BALB/c mice immunized with unconjugated BGG also caused some depression of the concentration of CRI in the anti-Ar antibodies subsequently induced in the C.AL-20 recipients. The results are similar to those obtained with 75 × 10^6 cells from nonimmune donors or donors immunized with KLH (Table I). Since the spleen cells transferred undoubtedly contained unprimed B cells with specificity for the Ar hapten group, these results may be attributable to the production of anti-Ar antibodies of BALB/c origin in the recipient mice. As already indicated, this would lower the relative proportion of anti-Ar antibodies with the CRI. The reduction was, however, far smaller than that induced by BALB/c cells primed with the Ar hapten group.

The average concentration of the CRI in group 1, Table III, in which the mice were irradiated but did not receive cells, is higher than that of group 1 in Table I, in which the mice were similarly treated. The explanation for this is not apparent but may be attributable to the small size of the group. In both instances, however, the transfer of cells from BALB/c mice immunized against the Ar hapten group caused almost complete suppression of the idiotype.

An additional set of experiments was carried out in which the splenic lymphocytes from BALB/c mice immunized with BGG-Ar were treated with anti-Thy-1.2 and complement before transfer to C.AL-20 recipients that had received 200 rads. The transfer of cells remaining after treating 20 × 10^6 BALB/c splenic lymphocytes caused complete inhibition of synthesis of the CRI in three of the four recipients and more than 90% inhibition in the other recipient, upon subsequent immunization with KLH-Ar (data not shown).

**Discussion**

The data presented indicate that the adoptive transfer of B cells, primed to a hapten but incapable of producing a specific idiotype, can inhibit completely the appearance of the idiotype that would otherwise have appeared upon immunization of the recipient animal with the same hapten group. The donors
Inhibition of CRI in C.AL-20 Mice by Adoptive Transfer of Spleen Cells from BALB/c Mice Immunized with BGG-Ar*

| Donor of cells transferred | No. of recipient mice | No. of cells transferred to each mouse | Nanograms anti-Ar Ab required for 50% inhibition$ |
|---------------------------|------------------------|---------------------------------------|-----------------------------------------------|
| None§                     | 5                      | None                                  | $30, 30, 40, 40, 60                           |
| BALB/c immunized with BGG¶ | 3                      | 10                                    | 150, 470, 850                                 |
|                           | 3                      | 50                                    | 31, 110, 690                                 |
|                           | 2                      | 75                                    | 600, 2,000                                   |
| BALB/c immunized with BGG-Ar~ | 5                      | 1                                     | 20, 60, 80, 100, 120                          |
|                           | 1                      | 10                                    | 1,500                                         |
|                           | 4                      | 10                                    | >5,000 to >13,000                            |
|                           | 1                      | 50                                    | 6,600                                         |
|                           | 4                      | 50                                    | >7,000 to >35,000                            |
|                           | 3                      | 75                                    | >4,500 to >8,500                              |

Footnotes as in Table I.

in these experiments were BALB/c and the recipients were C.AL-20 mice irradiated with 200 rads. C.AL-20 mice, which possess genes coding for the immunoglobulin heavy chains of the AL/N strain on a BALB/c background, produce anti-Ar antibodies with an idiotype (CRI) characteristic of the A strain, whereas the anti-Ar antibodies of BALB/c mice do not express this idiotype (7, 17–19). In nearly all recipient mice that received $10 \times 10^5$ cells or more the CRI was undetectable by a highly sensitive radioimmunoassay.

The adoptive transfer of specific idiotypes or spectrotypes is not novel, having previously been demonstrated in several laboratories (e.g., 1–5). The unexpected finding was the essentially complete suppression of the CRI. It is difficult to decide whether this can be explained simply by statistical considerations or whether some type of active suppression is involved. Suppression by idiotype-specific T cells seems, however, to be ruled out. First, the transferred cell populations were treated with anti-Thy-1.2 and complement. A second factor is that the donor mice were BALB/c whereas the recipients were C.AL-20 mice. Since BALB/c mice do not produce the CRI one would not expect them to synthesize idiotype-specific suppressor T cells. In addition, previous demonstrations of the production of idiotype-specific suppressor T cells have required the initial use of anti-idiotypic antibodies (3, 20), which were not employed in the present work.

In the first set of experiments, the BALB/c donors were immunized with KLH-Ar before the adoptive transfer; the C.AL-20 recipients, subsequently immunized with KLH-Ar, failed to produce the idiotype. It was conceivable that primed BALB/c donor mice generated helper T cells with specificity for idiotypes other than the CRI characteristic of anti-Ar antibodies of the A strain. (This assumes the existence of idiotype-specific helper cells.) It would have to be argued, in addition, that the treatment with anti-Thy-1.2 did not completely eliminate such helper T cells. Another possibility considered was
that the BALB/c donors which were primed with either KLH or KLH-Ar generated carrier-specific suppressor T cells which interfered with the activity of KLH-specific helper T cells in the C.AL-20 recipients. If the production of the CRI requires more "help" from T cells than the production of other idiotypes in the C.AL-20 strain, this might have selectively depressed the proportion of antibody molecules bearing the CRI. Again, the results obtained after treatment with anti-Thy-1.2 and complement would argue against this mechanism.

Nevertheless, in an effort to rule out these possibilities additional experiments were performed in which the BALB/c donors were primed with BGG-Ar rather than KLH-Ar and the C.AL-20 recipients were then immunized with KLH-Ar. This was done to obviate possible effects of KLH-specific T cells from the BALB/c donors. The results indicated that spleen cells from BALB/c donors immunized with BGG-Ar were capable of completely inhibiting the appearance of the CRI in C.AL-20 recipients subsequently immunized with KLH-Ar. In addition, the suppressive activity was insensitive to treatment with anti-Thy-1.2 and complement. The data appear to exclude a role for KLH-specific T cells.

Included among the control experiments were the adoptive transfers of cells from nonimmunized BALB/c mice or from BALB/c mice that had been immunized with unconjugated KLH or BGG. In each instance the C.AL-20 recipients produced substantial concentrations of anti-Ar antibodies bearing the CRI, indicating that graft-vs.-host reactions were not responsible for inhibition of idiotype synthesis. These experiments showed, in addition, that preimmunization of the donor mice to the hapten group was essential for marked suppression of the CRI in the C.AL-20 recipients. The results of these control experiments are consistent with the concept that inhibition is caused by B-cell dominance.

A number of factors are relevant to the complex question as to whether numerical superiority of B cells with specificity for anti-Ar antibodies lacking the CRI is solely responsible for the observed effect. Klinman has shown that the number of B cells specific for hapten increases less than 10-fold upon immunization (21). However, his immunization schedule was considerably less rigorous than ours. It is possible, therefore, that the number of memory cells may have been increased to a greater extent in our donor animals. If one assumes a B-cell precursor frequency for the CRI of 1 in 10,000, a relatively high value (22), then a normal recipient mouse would possess only ~10,000 B cells with receptors for the CRI. In addition, this number may have been reduced considerably as a result of irradiation with 200 R. It is thus difficult to assess the ratio of the two types of relevant B cells in the recipients, i.e., primary B cells with receptors for CRI and primed donor cells with anti-Ar receptors lacking the idiotype.

A factor that is probably more significant is the greater avidity of memory cells for antigen. A number of investigators have shown that primed cells are much more easily triggered than unprimed cells by antigen and that this reflects a higher avidity of such cells for antigen and/or an increased number of receptors (23–31); actually, the two factors may be interrelated since a high density of receptors would increase the possibility of multivalent attachment of the antigen. It was nevertheless surprising that the idiotype was not detectable in recipients of $10 \times 10^6$ or more lymphocytes from BALB/c donors primed with
the Ar hapten group. Perhaps the simplest hypothesis is that the very low concentration of CRI was due to a combination of numerical superiority of the donor cells lacking the CRI and their greater ease of triggering. Once established, the ratio of anti-Ar antibodies with and without the CRI might tend to be quite stable. This possibility is supported by the prolonged persistence during immunization of antibodies of a given molecular species (e.g., 32-36), although gradual changes are seen. In this context it is relevant that in the present experiments the suppressed state persisted for at least 6 mo in those mice which were tested.

The high sensitivity of the assay for idiotype indicates that the presence of secondary B cells can prevent essentially completely the expression of primary cells with receptors of the same specificity but of a different idiotype. A corollary conclusion is that clones of cells initially triggered in the immune response may tend to dominate the response for long periods of time. It is necessary, however, to consider the fact that the affinity of antibodies tends to increase during the course of the immune response (37-39). One might reconcile such findings with the present results on the basis that: (a) the type of inhibition we have observed would apply to antibodies of relatively low affinity; it has been found that anti-Ar antibodies bearing the CRI are somewhat lower in average affinity than the remainder of the anti-Ar population in the A/J strain (40). It would be relevant to determine whether the transfer of BALB/c B cells resulted in complete inhibition of the C.AL-20 anti-Ar response. This would be difficult to do with the system used here because BALB/c and C.AL-20 mice do not differ with respect to IgG1 allotype, and most anti-Ar antibodies of the A strain belong to this subclass (41). (b) The frequently reported increase in average affinity may reflect the preferential expansion of secondary clones of cells that were triggered by the initial antigenic stimulation; in our experiments secondary B cells were adoptively transferred into unprimed animals. In any event, the virtually complete suppression of an idiotype through B-cell dominance was rather unexpected and the possibility of B-cell-mediated suppression through an unknown, active mechanism cannot be excluded.

In the absence of direct evidence for active suppression one might postulate that the precursor cells committed to the CRI persisted in a dormant state throughout the period of the experiments. In the case of suppression with T cells or antiidiotypic antibodies such cells may be eliminated. A question under investigation is whether idiotype-specific suppressor T cells are generated in mice that have been suppressed initially by the transfer of hyperimmune B cells lacking receptors with the CRI.

Summary

Mice of the C.AL-20 strain, which express genes controlling CH regions of the AL/N strain on a BALB/c background, normally synthesize antibodies to the p-azophenylarsonate group (anti-Ar antibodies) with an idiotype characteristic of the A strain. The synthesis of the idiotype, as quantitated by a sensitive assay, can be completely inhibited by the transfer of leukocytes from BALB/c mice producing anti-Ar antibodies, which lack the idiotype. A number of
control experiments show that the inhibition is not attributable to suppressor T cells and that the synergistic action of such cells is not required. The results indicate that B-cell dominance, mediated by secondary cells, can completely prevent the expression of unprimed cells with receptors of the same specificity. It is uncertain whether this effect is due entirely to selective capture of antigen by the secondary cells, or whether some type of active suppression by B cells is involved.

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