# ANALYSIS OF THE RESPONSE OF B CELLS FROM CBA/N-DEFECTIVE MICE TO NONSPECIFIC T CELL HELP*

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B cell functional heterogeneity has been well studied over the last few years. It is now established that different B cell subsets mount productive antibody responses to different antigens (1–8), although the exact way in which responses to various antigens will be assigned to B cell subsets is still a matter of some controversy.

Particularly useful in these experiments has been the X chromosome-linked defect (*xid*) carried by the CBA/N mouse. A lesion in the *xid* gene results in a mouse with low response to certain antigens (9–13) and a complete absence of B cells able to respond to type 2 thymus-independent antigens such as trinitrophenylated Ficoll (1). These mice also lack B cells bearing the surface markers Lyb-3 (14) and Lyb-5 (15). Lyb-5$^-$ B cells from normal animals have been reported deficient in their ability to respond to thymus-dependent antigens such as sheep erythrocytes (SRBC)[1] when incubated with the normally stimulatory collection of factors present in the concanavalin A (Con A)-stimulated supernatants (Sn) of normal spleen cells. These B cells however, respond to SRBC in the presence of other factors (16).

This collection of observations suggests that CBA/N B cells have a specific defect in their ability to respond to the nonspecific factors in Con A Sn, but not to other types of T cell help. In the past, we have suggested that all thymus-dependent responses are dependent on such nonspecific T cell-derived factors, although there may be additional requirements, for example, for carrier-specific helper T cells (17). At first sight, this theory is contradicted by the data concerning CBA/N B cells, since these cells can indeed respond to antigen in the presence of helper T cells, but may not be driven by a collection of nonspecific helper factors.

The data in this manuscript represent our initial experiments aimed at investigating this contradiction. Our experiments confirmed that B cells carrying an X chromosome from CBA/N mice only were indeed defective in their ability to respond to SRBC driven by nonspecific helper factors. The cells were defective, however, in the factor-

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[1] *Abbreviations used in this paper:* BRBC, burro erythrocytes; Con A Sn, concanavalin A-stimulated spleen cell supernatant; KLH, keyhole limpet hemocyanin; MØ, macrophage; PFC, plaque-forming cells; SRBC, sheep erythrocytes.

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independent first stage of this response, in which division was initiated in normal B cells, but not in CBA/N-defective B cells, by the presence of SRBC alone, in the absence of helper factors. CBA/N B cells may therefore appear unresponsive to nonspecific factors, when in fact the defect in response lies entirely in the interaction between RBC antigens and B cells.

## Materials and Methods

*Mice.* C57BL/10.Sg Sn female and male mice were purchased from The Jackson Laboratory, Bar Harbor, Maine. CBA/N, (CBA/N × B10)$F_1$, and (B10 × CBA/N)$F_1$ mice were bred in our own facility from breeding pairs of CBA/N mice kindly provided by Dr. Diane Jacobs, SUNY at Buffalo, Buffalo, N. Y.

*Antigens.* SRBC, horse erythrocytes, and burro erythrocytes were obtained from single animals at Colorado Serum Co., Denver, Colo. Keyhole limpet hemocyanin (KLH) was purchased from Calbiochem-Behring Corp., San Diego, Calif.

*Immunization Procedures.* Animals were immunized for RBC-specific helper T cells with 0.2 ml of a 0.05% suspension of the appropriate RBC. 4 d later, helper T cells were isolated from the spleens of these animals (18). Helper T cells specific for KLH were primed by injection of 100 μg of KLH in 0.06 ml of complete Freund's adjuvant in the base of the tail. T cells were harvested 6–8 d later from inguinal and periaortic lymph nodes (19).

*Cell Preparations.* T cells were purified by passage by cell suspensions over nylon fiber columns (20). B cell and macrophage (MØ) suspensions were obtained by treatment of spleen cell suspensions with rabbit anti-T cell serum (21) and/or with a hybridoma anti-Thy-1 reagent (T24/40.7, kindly given to us by Dr. Ian Trowbridge, Salk Institute) followed by incubation with rabbit complement.

*Cell Culture.* Culture conditions were modified from those of Mishell and Dutton (8, 22). Cells were cultured in either 0.1 ml of medium in Costar 96-well microculture plates (3596; Costar, Data Packaging, Cambridge, Mass.) or in 0.5 ml of medium in Costar 3524 24-well plates. Antigen concentrations were: RBC antigens, 0.05%, KLH, 5 μg/ml.

*Preparation of Con A Sn.* Spleen cell suspensions were cultured at $10^7$ cells/ml for 24 h with 4 μg/ml Con A. Supernatants were then harvested, absorbed with Sephadex G-200 to remove residual Con A, filtered, and stored in aliquots of −20°C until used (23).

*Quantitation of Helper Activity.* RBC-specific helper activity was measured by titrating the T cells to be assayed into cultures containing constant numbers of B cells, MØ, and the RBC antigen (24). 4 d later, plaque-forming cells (PFC) per culture against the pertinent RBC were measured and plotted against the number of T cells added to the cultures. To quantitate the helper activity, a straight line was fit to the initial, linear portion of this titration and the slope of the line was taken as a relative measure, expressed as PFC/$10^6$ T cells/culture ± standard error of the helper activity of the T cell preparation on the B cell and MØ preparation used (24).

Bystander helper activity, i.e., the ability of T cells to respond to one antigen and help B cells respond to an unrelated RBC antigen, was measured in a similar fashion. Thus, KLH-primed helper T cells were titrated into cultures containing constant numbers of B cells, MØ, SRBC, and KLH. After 4 d, PFC responses to SRBC was measured. These were plotted and quantitated as described previously (25). Cultures to control for background SRBC-specific helper activity in our KLH-primed T cell preparations were set up and assayed as described above, except that KLH was omitted from the cultures.

*Determination of B Cell Precursor Frequencies.* RBC-specific B cell precursor frequencies were determined by limiting dilution analysis as previously described (7, 8). Briefly, microcultures containing antigen and excess T cell help (primed T cells or Con A Sn) were prepared. B cells were titrated into these cultures in a range where not all cultures responded. The log of the fraction of microcultures failing to respond was plotted vs. the number of B cells added to yield a linear plot. The frequency of RBC-responsive B cells in different populations was calculated assuming Poisson distribution laws by computer-aided modified linear regression analysis of the data.

*Assay of PFC.* Anti-RBC PFC were assayed either directly in the microculture cells (26) or in the case of 0.5-ml cultures, by the slide modification (22) of the hemolytic plaque assay.

## Results

*Effects of Various Forms of T Cell Help on Defective and Normal B Cells.* Others have shown that in the presence of Con A Sn and RBC antigen, B cells from normal mice are induced to become plasma cells secreting anti-RBC antibodies (23, 27–30). Because it has been reported that CBA/N-like B cells have a defective response of this and related types (16, 31), we wished to confirm this result. The results of an experiment comparing the ability of (CBA/N × B10)$F_1$ female and male B cells to respond to SRBC in the presence of Con A Sn or SRBC-primed T cells, from (CBA/N × B10)$F_1$ female mice, are shown in Fig. 1. Both (CBA/N × B10)$F_1$ male and female B cells responded to SRBC when SRBC-primed T cells were titrated into the cultures. A consistent observation in our experiments, however, was that (CBA/N × B10)$F_1$ female B cells always responded about twice as well to SRBC as a comparable number of male B cells. (CBA/N × B10)$F_1$ female B cells responded well to SRBC driven by Con A Sn, but (CBA/N × B10)$F_1$ male B cells did not yield antibody-secreting cells under such conditions.

*Production of Con A Sn by Defective and Normal Cells.* Because we observed a lack of responsiveness to Con A Sn by the (CBA/N × B10)$F_1$ male B cells, we were interested in the ability of cells from defective animals to produce an active preparation of Con A Sn. Con A Sn was therefore prepared from female or male (CBA/N × B10)$F_1$ spleen cells and then tested for helper activity on female (CBA/N × B10)$F_1$ B cells responding to SRBC. The results are shown in Fig. 1. Female and male (CBA/N

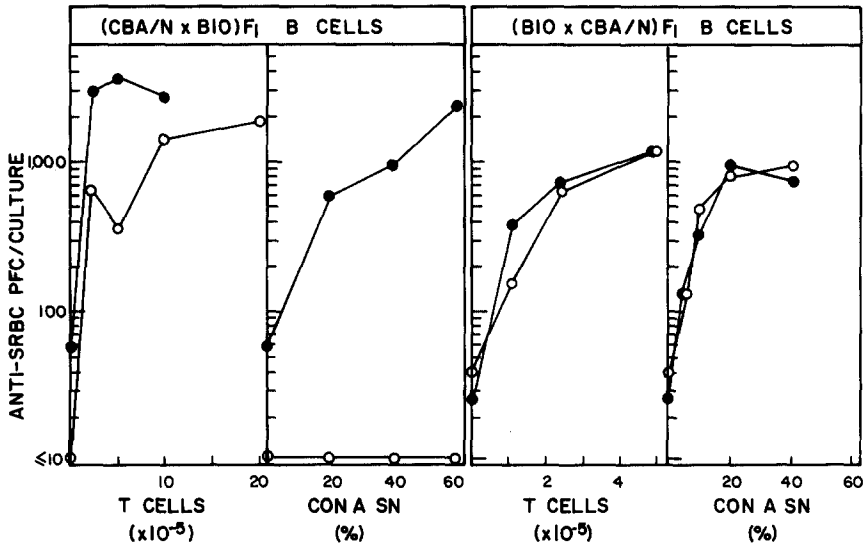


FIG. 1. (CBA/N × B10)$F_1$ male defective B cells did not respond to the helper activity in Con A Sn. Groups of three 0.5-ml cultures were prepared containing 1.5 × $10^6$ B cells and MØ from the indicated $F_1$ cross. Cultures received SRBC as antigen and either various numbers of female SRBC-primed T cells or various concentrations of Con A Sn as T cell help. After 4 d, the cultures in each group were pooled and assayed for anti-SRBC PFC. ●, female B cells; ○, male B cells. The (CBA/N × B10)$F_1$ data are from one of three similar experiments, whereas the (CBA/N × B10)$F_1$ data are from one experiment.

B10)$F_1$ splenocytes produced Con A Sn with almost equivalent helper activity in this assay. The ability of both female and male (CBA/N × B10)$F_1$ SRBC-primed T cells to support female (CBA/N × B10)$F_1$ B cell responses to SRBC is also shown in Fig. 2. Quantitative analysis of the response showed that female (CBA/N × B10)$F_1$ T cells had about twofold higher helper activity than male T cells.

*Nonspecific Bystander Help Did Not Activate Male (CBA/N × B10)$F_1$ B Cells.* In previous experiments we and others (21, 25, 27) have shown that T cells primed to protein antigens such as KLH or human gamma globulin help B cells respond to bystander RBC antigens, probably by means of nonspecific factors, provided both the priming and RBC antigens were added to the cultures. Because such a bystander response seems to be driven by nonspecific factors similar to those found in Con A Sn, (CBA/N × B10)$F_1$ B cells from mice of each sex should behave in a predictable fashion in such a system, i.e., female cells should respond and male cells should not. Therefore, T cells from both female and male (CBA/N × B10)$F_1$ mice were primed to KLH and then titrated into cultures containing female or male (CBA/N × B10)$F_1$ B cells, SRBC and KLH. The anti-SRBC response was measured 4 d later. As illustrated in Table I, T cells from both female and male (CBA/N × B10)$F_1$ mice could deliver bystander help to female (CBA/N × B10)$F_1$ B cells. In contrast, they were ineffective when assayed with B cells from the defective male mice. Control experiments showed that both male and female B cells could respond to SRBC in the presence of SRBC-primed T cells. In other control experiments (data not shown), both male and female (CBA/N × B10)$F_1$ B cells were shown to respond to SRBC in the presence of bystander helper T cells, thus demonstrating that defect we were measuring was not Y chromosome-linked, but dependent on the CBA/N X chromosome.

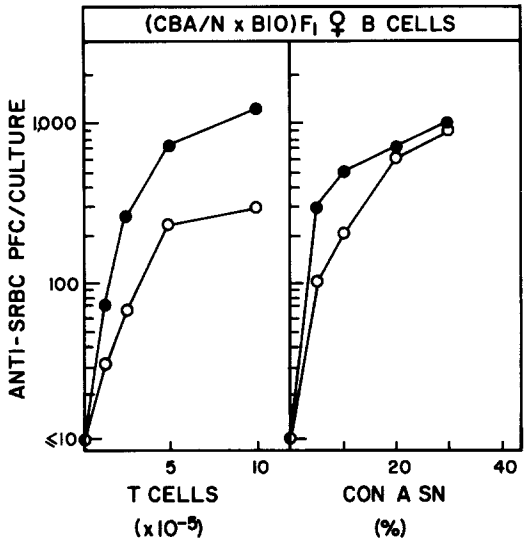


FIG. 2. Spleen cells from (CBA/N × B10)$F_1$ males were capable of producing active Con A Sn. Groups of three 0.5-ml cultures were prepared containing 1.5 × $10^6$ (CBA/N × B10)$F_1$ female B cells and MØ. Cultures received SRBC as antigen and either various numbers of female (●) or male (○) SRBC-primed T cells or various concentrations of Con A Sn from each source as help. After 4 d, the cultures in each group were pooled and assayed for anti-SRBC PFC. The results shown are from one experiment, typical of three.

TABLE I

*(CBA/N × B10)F$_1$ Male B Cells Did Not Respond to Nonspecific Bystander Helper Activity*

| Cells in culture | | T cell priming | Antigen in culture | Helper activity (anti-SRBC PFC/$10^6$ T cells/culture + SE) |
|---|---|---|---|---|
| B cell + MØ | T cell | | | |
| ♀ | ♀ | KLH | SRBC + KLH | 550 ± 180 |
| | ♂ | KLH | SRBC + KLH | 920 ± 180 |
| ♂ | ♀ | KLH | SRBC + KLH | 16 ± 4 |
| | ♂ | KLH | SRBC + KLH | 16 ± 4 |
| ♀ | ♀ | SRBC | SRBC | 1,629 ± 193 |
| | ♂ | SRBC | SRBC | 784 ± 331 |
| ♂ | ♀ | SRBC | SRBC | 740 ± 94 |
| | ♂ | SRBC | SRBC | 472 ± 177 |

*RBC Antigens Did Not Activate B Cells with the CBA/N Defect.* In past experiments, the response of B cells to RBC-bound antigens has been divided into two stages. In the first stage, RBC-bound antigens alone were shown to stimulate B cells in the apparent absence of T cell help. T cells or nonspecific T cell factors were then required to complete differentiation and probably to stimulate division (28, 29). Because male CBA/N F$_1$ B cells responded abnormally, we wished to determine which portion of the response was aberrant.

The initial phase of the response can be studied in isolation by culturing B cells with or without RBC antigen for 24 h in bulk cultures, and then assaying the frequency of RBC-specific precursor B cells by limiting dilution in the presence of antigen and sufficient T cell help, in this case excess RBC-primed helper T cells. Normal B cells have been tested in such an assay, and the presence of antigen during the 24-h bulk culture has been shown to cause a two- to fourfold increase in antigen-specific B cell precursors (17).

An experiment of this type was therefore carried out using (CBA/N × B10)F$_1$ male and female B cells. BRBC were chosen as the antigen, because SRBC often cross-react with antigens in fetal calf serum, making it difficult to omit SRBC-related antigen from the first 24-h bulk culture. Thus B cells of different types were bulk-cultured for 24 h with or without BRBC, and with or without BRBC-primed T cells. At this point, the B cells were titrated into microcultures containing BRBC and BRBC-primed T cells. The results of one such experiment, representative of three, are shown in Table II.

BRBC-reactive B cells in female (CBA/N × B10)F$_1$ populations increased in frequency two- to fourfold in the presence of BRBC for 24 h whether or not BRBC-reactive T cells were present. There was a slight increase in BRBC-reactive B cell frequency in the presence of BRBC-primed T cells alone, perhaps because of contaminating B cells in the T cell population. In contrast, incubation with antigen did not cause an increase in BRBC-reactive B cell precursor frequency in male (CBA/N × B10)F$_1$ B cells. An effect on these cells was only seen if both BRBC and BRBC-specific helper T cells were present during the 24-h bulk culture. This effect was small, but significant, and showed that cells carrying the CBA/N defective gene alone could not be activated by RBC antigens in the absence of antigen-specific T cell help. This

TABLE II

*(CBA/N × B10)F$_1$ Male Defective B Cells Did Not Proliferate in the Presence of RBC Antigens Alone*

| B cell source | Conditions during the first 24 h* | | Anti-BRBC precursor frequency/$10^5$ cells§ |
|---|---|---|---|
| | BRBC‡ | BRBC-primed T cells | |
| (CBA/N × B10)F$_1$ ♀ | + | + | 4.1 ± 1.0 |
| | + | − | 3.0 ± 1.0 |
| | − | + | 1.5 ± 0.2 |
| | − | − | 0.9 ± 0.2 |
| (CBA/N × B10)F$_1$ ♂ | + | + | 1.4 ± 0.3 |
| | + | − | 0.8 ± 0.3 |
| | − | + | 0.6 ± 0.3 |
| | − | − | 0.7 ± 0.3 |

* The cells were cultured at $10^7$/ml in 0.5-ml cultures with or without BRBC as antigen and with or without $5 \times 10^6$ BRBC-primed T cells.

‡ SRBC (a non-cross-reacting antigen) were added to the cultures that did not receive BRBC to maintain cell viability.

§ After 24 h, the B cells in each group were harvested and replated in microwells at various concentrations in the presence of BRBC and $1 \times 10^5$ BRBC-primed T cells. After an additional 4 d, 12 individual cultures for each B cell concentration were assayed for anti-BRBC PFC. The results shown are the mean precursor frequency ± SEM of three experiments.

suggested that the defect in CBA/N B cell responses to RBC antigens in the presence of Con A Sn might be in the initial stage of this response.

## Discussion

The results in this paper demonstrate the failure of male (CBA/N × B10)F$_1$ B cells to respond to RBC-bound antigens in the presence of nonspecific T cell helper factors. B cells that expressed the CBA/N defect could not be stimulated to mount a PFC response to SRBC when driven either by helper factors present in Con A Sn or by bystander helper T cells, in the shape of KLH-stimulated, KLH-primed T cells.

These experiments confirm results reported by some others (16, 31), with the additional observation that bystander help is also not effectively received by CBA/N-defective B cells. This supports the idea, which has been suggested in the past, that bystander help is mediated by Con A Sn-like nonspecific helper factors. Some previous investigators (10, 32) have concluded that CBA/N B cells are responsive, albeit less than normal controls, to the helper activities found in Con A Sn and similar preparations. However, their results could be a result of activation of contaminating T cells in the B cell preparations, which we have shown can lead to confusing results (33).

As other laboratories have done, we checked the helper activity of T cells from CBA/N F$_1$ male and female mice. As has been previously observed, no specific defect in helper activity of cells from male mice was noted, though usually they were about half as active as comparable numbers of cells from female animals in any given assay. Thus, it was shown that active Con A Sn production and KLH-specific bystander helper activity could be demonstrated in male cells when assayed on female B cells. SRBC-specific helper T cells could be primed in male animals, as could KLH-specific

helper cells adequate for a trinitrophenyl-KLH response (results not shown). Bottomly et al. (34) previously reported that CBA/N $F_1$ male mice lack idiotype-specific helper T cells. Because it is not at all clear that such helpers are active in the IgM anti-SRBC responses studied in this paper, it is perhaps not surprising that their absence did not affect our results.

Dutton (28) and Schimpl et al. (29) have split the anti-RBC response into two stages by varying the time at which T cell help was added. The first stage, which lasts for 24–48 h, is apparently T cell independent, and probably involves the induction of at least one round of division in antigen-specific B cells, although recruitment of B cells into an antigen-reactive state by antigen plus MØ products might also occur during this period. The second stage of response is marked by an absolute requirement for nonspecific helper factors such as those found in Con A Sn, or antigen-specific T cells, in order for anti-RBC PFC to appear. We therefore designed an experiment to determine whether CBA/N $F_1$ male B cells were aberrant in the first, apparently T cell independent, stage of this response. Our results showed clearly that they were, because these defective B cells could only be activated if both antigen and helper T cells were present. Control $F_1$ female B cells could divide in the presence of antigen alone. Interestingly, the increase in SRBC-specific B cell precursor frequency of $F_1$ male cells was never more than twofold, even in the presence of both antigen and helper T cells. $F_1$ female cells under similar conditions showed fourfold increases. This difference may, again, reflect the effects of the defective CBA/N gene.

These results suggest, but certainly do not prove, that B cells bearing the CBA/N-defective gene may be unable to respond to nonspecific helper factors because RBC are unable to activate the B cells, not because of some intrinsic inability of the B cells to respond to such mediators. They suggest that if B cell responses could be initiated by some other means, for example by antigen-specific T cells in the presence of antigen, then the rest of the B cell response may be satisfactorily driven by nonspecific factors. Our future experiments will be aimed at testing this hypothesis.

To conclude that the results in this paper are a result of CBA/N phenotype is possible only after eliminating Y chromosome effects or the consequences of X chromosome gene doses. These alternative possibilities have become a concern since a recent observation (32) that the acceptor molecule for a nonspecific T helper activity is X-linked. All experiments done in this paper were repeated using nondefective B cells from female and male (B10 × CBA/N)$F_1$ mice. In these experiments (not shown here), the activities of female and male B and T cells were equivalent. Thus the defect demonstrated in male (CBA/N × B10)$F_1$ B cells is a result of the X-linked CBA/N-defective geneotype.

There is considerable interest over the question whether CBA/N $F_1$ male B cells have their counterpart in the normal mouse. This is certainly well documented as far as surface phenotype is concerned, because work by a number of laboratories has shown that normal mice contain Lyb-$5^-$, $IgD^-$, $MIs^-$ cells bearing high amounts of Ia and immunoglobulin that seem analogous to those found in mice bearing the CBA/N-defective gene. Although the experiments of this paper have confirmed the idea of others that CBA/N $F_1$-defective B cells do not respond properly to RBC driven by nonspecific helper factors, this has not been the case when we examined CBA/N-like B cells in normal animals. We have shown that high *I-A*, high surface immunoglobulin-bearing B cells from normal mice respond as well to SRBC driven by Con A

Sn as do their low *I-A,* low surface immunoglobulin counterparts (7, 8). In contrast, Ahmed and Scher (16) have found that Lyb-5$^-$ cells (which may be the same subset as that which bears high surface *I-A* and immunoglobulin) are unresponsive to RBC plus Con A Sn. This dichotomy has yet to be resolved. Considering results from our own laboratory, however, cells with apparently the same surface phenotype do not always behave identically, perhaps because the CBA/N gene defect prevents certain types of response by B cells bearing it, although such responses would be allowed in normal B cells at that maturational stage.

## Summary

We have investigated the induction of antibody responses to erythrocyte (RBC)-bound antigens in the (CBA/N × B10)$F_1$ mouse. Male B cells, which express the CBA/N defect, were shown to be unresponsive to RBC antigens when the delivered T cell helper activity was solely nonspecific. Thus we demonstrated that defective B cells did not respond to concanavalin A supernatants or bystander helper activity, in spite of the fact that CBA/N-defective mice could produce these T cell activities. The defective B cell did respond to RBC-bound antigen in the presence of RBC-primed T cells, although the magnitude of this response was usually twofold less than normal controls. The insensitivity of CBA/N defective B cells to nonspecific T cell helper activities seemed to involve at least the inability of RBC antigens to activate defective B cells in the absence of antigen-specific T cell help.

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