CELLULAR AND MOLECULAR REQUIREMENTS FOR X-LINKED, HAPTEN-SPECIFIC B-CELL BLOCKADE IN CBA/N MICE

BY B. MERCHANT, H. SNIPPE, E. F. LIZZIO, AND J. K. INMAN

(From the Immunohematology Branch, Division of Blood and Blood Products, Bureau of Biologics, Food and Drug Administration, and the Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20014)

CBA/N mice, a mutant CBA subline, are of particular interest for studies of the requirements for B-cell activation since they harbor an X-linked B-cell defect (1). The defect is characterized by low levels of circulating IgM, reduced numbers of splenic B cells, and a total failure of existing B cells to mount immune responses to certain thymic-independent antigens, e.g., haptenated Ficoll derivatives (2-5). Those B cells present in CBA/N spleens are characterized by a generally increased density of cell surface IgM (6). In addition, CBA/N mice have an abnormally high ratio of mu chain to delta chain homolog on their B-cell surfaces (7). In this respect, the Ig receptor density of CBA/N B cells appears to resemble the Ig density on B cells of neonatal and very young mice (8). Since the usual progression from mu chain to delta chain homolog expression on these cell surfaces is greatly delayed in CBA/N mice and in the hybrid male progeny of CBA/N females, Scher et al. have proposed that the CBA/N defect might represent an arrest in B-cell maturational development (6, 7).

CBA/N mice and their affected hybrid male offspring are also profoundly sensitive to selective B-cell blockade (9). Several other animal models exist in which the injection of a haptenated thymic-independent antigen can block a subsequent response to a thymic-dependent antigen bearing the same hapten (10-14). The marked blockade susceptibility of CBA/N mice is transmitted via the X chromosome, since all the male hybrid offspring of CBA/N females are affected. The hybrid female offspring are not affected at dosage levels of haptenated polysaccharide up to three orders of magnitude above those which can yield virtually total hapten-specific blockade both in the CBA/N parents and in the hybrid males (9).

The present study was designed to explore the cellular and molecular conditions necessary for production of this selective blockade. Several haptenated polysaccharide agents were examined for their relative effectiveness in producing blockade, and a wide range of efficiencies was observed. In addition, the effect of prior immune status on blockade susceptibility was investigated. The generation of hapten-specific immune memory to a thymic-dependent antigen virtually abolishes the inherent susceptibility of CBA/N mice to selective hapten-polysaccharide-mediated blockade. Moreover, the imposition of
blockade on secretory B cells in defective mice appears to have little if any effect on their subsequent development of B memory cell populations.

Materials and Methods

Animals. CBA/N, C3H/HeN, CBA/CaHN-T6, and F, hybrid mice derived from these inbred strains were obtained from the Small Animal Production Section of the National Institutes of Health, Bethesda, Md. The F, hybrids were prepared by breeding CBA/N female mice with C3H/HeN or with CBA/CaHN-T6 males. All mice used were between 6 and 15 wk of age. The CBA/N mice (formerly designated CBA/HN) are a mutant subline of CBA mice, and their origin has been previously described (1).

Immunogens. Keyhole limpet hemocyanin (KLH) was purchased from Schwarz/Mann, Div. Becton, Dickinson & Co., Orangeburg, N. Y. Ficoll, a synthetic polymer of sucrose with an average mol wt of 400,000, was obtained from Pharmacia Fine Chemicals, Piscataway, N. J. Pneumococcal polysaccharide, type III (SIII), was a gift from Dr. Phillip J. Baker, National Institute of Allergy and Infectious Diseases. The enlarged hapten, N-(2,4-dinitrophenyl)-β-alanylglycylglycine, (DNP), was synthesized as its tert-butyloxycarbonyl hydrazide as previously described (15). This reagent was converted to its reactive acyl azide (16) and coupled to N-(2-aminoethyl)-carbamylmethylated Ficoll (AECM-Ficoll) as described by Inman (17). The pH of the reaction mixture was adjusted to ~8.5 with 1 N KOH after addition of the hapten azide. The degree of conjugation was calculated from dry weight and optical absorbance measurements (17) to yield DNP25-AECM,Ficoll, or simply, "DNP25-Ficoll." The subscript refers to the average number of haptenic groups coupled to 400,000 daltons of the original Ficoll.

A portion of the naturally occurring carboxyl functions of pneumococcal polysaccharide, SIII, was converted to N-(2-aminoethyl) amide groups, and this modified polysaccharide was then conjugated with the reactive DNP azide as previously described (9). Spectrophotometric analysis (17) revealed 52 DNP groups bound to 400,000 daltons of polysaccharide. This product was designated DNP52-SIII.

DNP22-KLH was prepared as described by Inman, et al. (16). Here, subscripts refer to the number of haptenic groups per 100,000 daltons of original KLH. Ficoll, conjugated with N-e-dinitrophenyl-l-lysine (with no tripeptide), was a gift from Dr. Philip R. B. McMaster, Bureau of Biologics. It was prepared by a cyanuric chloride coupling procedure which McMaster et al. have published (18). For convenience, this smaller-hapten derivative is designated as dpn2,Ficoll. All antigens and blocking agents were injected intraperitoneally (i.p.).

Hapten-Conjugated Erythrocytes. Sheep erythrocytes (SRC) were obtained in sterile Alsever's solution from the National Institutes of Health Animal Production Unit. SRC, pooled from six to eight sheep, were conjugated with the tripeptide-enlarged DNP hapten under optimal conditions according to the method of Inman et al. (16).

Cell Suspensions. Spleens were obtained from mice at sacrifice, minced in cold Hanks' balanced salt solution, and cell aggregates were disrupted by aspiration through a Pasteur pipette. The cell suspensions were passed through a thin layer of glass wool to remove fibrous connective tissue, washed in Hanks' balanced salt solution, and counted on a model B Coulter counter (Coulter Electronics Inc., Hialeah, Fla.).

Plaque-Forming Cell Assays. Suspensions of spleen cells from individual mice were assayed for DNP-reactive antibody-secreting cells by a modification of the Jerne hemolytic plaque technique as previously reported (19). All recorded plaque-forming cell (PFC) values have been corrected for coexisting direct PFC background against SRC. Data have been expressed as the mean log10 PFC/spleen ± SEM. Student's t test was employed to evaluate differences between groups of mice. Differences were considered to be significant when P values of < 0.05 were obtained.

Results

The Effect of Varying Hapten Derivatization Levels on the Efficacy of Specific Hapten-Polysaccharide-Mediated Blockade. AECM25-Ficoll was conjugated

Abbreviations used in this paper: AECM, Aminoethylcarbamylmethyl; dpn, the 2,4-dinitrophenyl hapten; DNP, the tripeptide-enlarged hapten, N-(2,4-dinitrophenyl)-β-alanylglycylglycine; i.p., intraperitoneally; KLH, keyhole limpet hemocyanin; PFC, plaque-forming cells; SIII, pneumococcal polysaccharide, type III; SRC, sheep erythrocytes.
with the tripeptide-enlarged DNP hapten at each of three derivatization levels, and these preparations were compared in dose-response experiments for their capacity to produce hapten-polysaccharide-mediated selective B-cell blockade. DNP_{52}-SIII and small hapten dn~5-Ficoll were also employed in order to evaluate their blockade-invoking capacity. The data obtained are presented in Fig. 1. The normalized dose-response curves show the relative capacity of these haptenated polysaccharide agents to block the 4th-day direct PFC response to DNP_{22}-KLH in CBA/N mice.

DNP_{59}-Ficoll was the most effective blockading agent. It produced roughly 90% suppression of 4th-day direct PFC after injection of as little as 100 pg. Due to individual mouse variation, the first statistically significant blockade with DNP_{59}-Ficoll occurred at the 10-ng dosage level. This still amounted to a 5,000-fold net "signal advantage" per unit of weight over the 50 μg of DNP_{22}-KLH which was successfully blockaded. As previously observed in normal mouse strains by Desaymard and Waldmann (20), and Desaymard et al. (21), reducing the derivatization level of DNP-polymers profoundly reduced their efficiency in producing hapten-specific blockade. Also, dn~5-Ficoll was particularly inefficient as a blockading agent. This implies that the tripeptide-enlarged form of the DNP hapten confers a more stable interaction with Ig receptors on target B cells, thus augmenting the blockade phenomenon. DNP_{52}-SIII, a linear saccharide polymer, was intermediate between DNP_{59}-Ficoll and DNP_{49}-Ficoll in its blockade-inducing effectiveness. This indicates that blockade is not produced exclusively by multi-branched polymers such as the haptenated Ficoll derivatives.
TABLE I
Failure of AECM-Ficoll to Produce Blockade of the 4th Day Direct PFC Response to DNP_{22}-KLH in CBA/N x C3H/HeN F_1 Mice

| Group number* and sex | Blocking agent± | Immunizing antigen | Mean log direct DNP-reactive PFC/spleen ± SE (geometric mean) |
|-----------------------|-----------------|--------------------|---------------------------------------------------------------|
|                       |                 |                    | Experiment A | Experiment B       |
| I. Male               | AECM_{120}-Ficoll | –                  | ND§          | 2.39 ± 0.05 (244) |
| II. Male              | –               | 50 µg DNP_{22}-KLH | 3.61 ± 0.11  | 3.19 ± 0.19 (1,548)|
|                       |                 |                    | (4,107)      |                   |
| III. Male             | AECM_{120}-Ficoll | 50 µg DNP_{22}-KLH | 3.78 ± 0.25|| 3.02 ± 0.13|||
|                       |                 |                    | (6,047)      | (1,046)           |
| IV. Female            | AECM_{120}-Ficoll | –                  | ND           | 3.54 ± 0.03 (3,496) |
| V. Female             | –               | 50 µg DNP_{22}-KLH | 4.47 ± 0.14  | 4.11 ± 0.14 (12,983) |
|                       |                 |                    | (29,279)     |                   |
| VI. Female            | AECM_{120}-Ficoll | 50 µg DNP_{22}-KLH | 4.33 ± 0.41¶ | 4.25 ± 0.07** (17,787) |
|                       |                 |                    | (21,222)     |                   |

* Four or five mice per group.
± 500 µg per mouse in Experiment A and 1 mg per mouse in Experiment B, always at −1 h.
§ ND, not determined.
|| Does not differ significantly from group II of the corresponding experiment; in both instances P ≥ 0.20.
¶ Does not differ significantly from group V; P ≥ 0.30.
** Does not differ significantly from group V; P ≥ 0.10.

Attempts to Blockade with Nonhaptenated Ficoll. In principle, blockade of B-cell activation by DNP-Ficoll might occur directly through its specific interaction with surface Ig receptors. Or, DNP-Ficoll might block by interaction of its polysaccharide moiety with some disparate surface component specialized to receive signals from polysaccharides or other polymeric entities. According to this latter alternative, Ig receptors would serve only to concentrate haptenated polysaccharide agents at the B-cell surface, with the carrier polysaccharide then delivering the critical signal to the defective cell. A preliminary test of this hypothesis was attempted by injecting 500 µg of AECM_{120}-Ficoll into male or female hybrid mice just 1 h before immunizing them with 50 µg of DNP_{22}-KLH. Our findings are presented in Table I. As often occurs, the 4th-day direct splenic PFC responses to DNP_{22}-KLH were substantially higher in the female than in the male hybrid mice in both experiments A and B. However, AECM_{120}-Ficoll did not appreciably reduce the response to DNP_{22}-KLH in male or female hybrid mice in either experiment. A further test was carried out in parental CBA/N mice using AECM_{85}-Ficoll. Here the preliminary injection of this polysaccharide carrier was given 4 h before primary immunization with DNP_{22}-KLH, and the AECM_{85}-Ficoll dosage was increased to 2 mg (Table II). Although this latter dose represents a 200,000-fold higher dosage of Ficoll than the 10 ng of DNP_{85}-Ficoll which provided significant blockade in Fig. 1, no indication of DNP-reactive PFC blockade could be detected.

Further experiments were performed to test whether blockade might be achieved by employing both the hapten and the Ficoll carrier signals separately. The results are provided in Table III. Free tripeptide-enlarged DNP hapten was
TABLE II
Failure of AECM₅₀-Ficoll to Blockade the 4th Day Primary Immune Response to DNP₂₂-KLH in CBA/N Mice

| Group number* | Blocking agent given at -4 h | Immunizing agent given at time zero | Mean log direct DNP-reactive PFC/spleen ± SE (geometric mean) |
|---------------|------------------------------|------------------------------------|---------------------------------------------------------------|
| I.            | -                            | 50 µg DNP₁₁-KLH                    | 3.53 ± 0.11 (3,392)                                           |
| II.           | 2 mg AECM₅₀-Ficoll           | 50 µg DNP₁₁-KLH                    | 3.57 ± 0.12‡ (3,776)                                          |
| III.          | 1 mg AECM₅₀-Ficoll          | 50 µg DNP₁₁-KLH                    | 3.48 ± 0.15‡ (3,050)                                          |
| IV.           | 500 µg AECM₅₀-Ficoll         | 50 µg DNP₁₁-KLH                    | 3.45 ± 0.09‡ (2,820)                                          |
| V.            | 250 µg AECM₅₀-Ficoll         | 50 µg DNP₁₁-KLH                    | 3.71 ± 0.15‡ (5,214)                                          |

* Five mice per group.
‡ Does not differ significantly from group I; P ≥ 0.15.

TABLE III
Chemical Attachment of Hapten and Ficoll Required for Effective Blockade of Direct PFC Response to DNP₂₂-KLH in CBA/N Mice

| Group number* | Blocking agents‡ | Mean log direct DNP-reactive PFC/spleen ± SE (geometric mean) |
|---------------|-----------------|---------------------------------------------------------------|
|               | First           | Second                                                        |
| I.            | -               | 10 µg DNP₁₁-Ficoll                                           | 4.50 ± 0.05 (31,761)                                         |
| II.           | -               | 0.2 ml 10⁻³M DNP-hydrazide                                   | 4.46 ± 0.15§ (29,969)                                         |
| III.          | 100 µg AECM₅₀-Ficoll | 0.2 ml 10⁻³M DNP-hydrazide                           | 4.53 ± 0.03§ (33,417)                                         |
| IV.           | 1 mg AECM₅₀-Ficoll      | 0.2 ml 10⁻³M DNP-hydrazide                                | 4.63 ± 0.13¶ (42,312)                                         |
| V.            | 500 µg AECM₅₀-Ficoll    | 10 µg DNP₁₁-Ficoll                                          | 3.00 ± 0.12$ (991)                                           |

* Five CBA/N female mice per group; all mice in all groups were immunized with 50 µg of DNP₂₂-KLH, i.p.
‡ First and second blocking agents given 10 min apart at -2 h before immunization with DNP₂₂-KLH.
§ Differs significantly from group I; P ≤ 0.005.
¶ The tripeptide-enlarged free hapten, N-(2,4-dinitrophenyl)-β-alanylglycylglycine Boc-hydrazide.
$ Does not differ significantly from group I; P ≥ 0.10.

ineffective by itself in producing hapten-specific blockade. Free hapten and free Ficoll carrier administered concomitantly were also ineffective, even when each was employed at a 100-fold higher concentration (group V) than its effective concentration when covalently combined (group II). In addition, 500 µg of
HAPten-SPECIFIC, B-CELL BLOCKADE IN CBA/N MICE

Fig. 2. Sustained blockade in CBA/N mice given DNP$_{59}$-Ficoll at various junctures before immunization with DNP$_{22}$-KLH. Groups of 5 mice each received 10 $\mu$g of DNP$_{59}$-Ficoll i.p. at intervals of up to 14 days before i.p. immunization with 50 $\mu$g of DNP$_{22}$-KLH. Splenic DNP-reactive direct PFC were determined 4 days after DNP$_{22}$-KLH immunization. Note that the degree of responsiveness is shown on a log scale.

AECM$_{120}$-Ficoll was ineffective in preventing the blockade imposed by only 10 $\mu$g of DNP$_{59}$-Ficoll. Accordingly, our findings indicate that the hapten and the carrier Ficoll must be chemically joined in order for hapten-specific blockade to occur.

Temporal Requirements for Production of Specific Hapten-Ficoll-Mediated Blockade. CBA/N mice were given 10 $\mu$g of DNP$_{59}$-Ficoll at various intervals before immunization with DNP$_{22}$-KLH and their direct splenic PFC responses were measured 4 days after immunization. The findings are depicted in Fig. 2 which discloses that the blockade phenomenon is obtained at least as efficiently with DNP$_{59}$-Ficoll delivered at $-24$ h as it is when the blockading agent is given at the time of immunization. DNP$_{59}$-Ficoll delivered at successively longer time intervals before immunization was progressively less effective in causing the blockade. When delivered 14 days before DNP$_{22}$-KLH immunization, 10 $\mu$g of DNP$_{59}$-Ficoll reduced the mean 4th-day PFC response to 18% of that in the control mice. Sufficient individual variation occurred in this group, however, so that the mean PFC reduction was not statistically significant. Thus, it appears that some mice have largely escaped from blockade after 14 days, but in others, the blockade remains relatively intact.

In another set of experiments, DNP$_{59}$-Ficoll was given at intervals ranging up to 48 h after immunization with 50 $\mu$g of DNP$_{22}$-KLH. The findings provided in Table IV, show that significant blockade was obtained in both CBA/N and F$_1$ male hybrid mice when DNP$_{59}$-Ficoll was given up to 4 h after DNP$_{22}$-KLH immunization. The findings also suggest that hybrid male mice may be susceptible to blockade for a longer time after immunization than their CBA/N parents.
### Table IV

**Blockade of the Splenic PFC Response to DNP<sub>22</sub>-KLH by Post-Immunization Injection of DNP<sub>59</sub>-Ficoll**

| Group number* | 50 µg DNP<sub>22</sub>-KLH given at time | 10 µg DNP<sub>59</sub>-Ficoll given at time | Mean log direct DNP-reactive PFC/spleen ± SE (Geometric Mean) [Percent of Control Response] |
|---------------|-----------------------------------------|---------------------------------------------|-----------------------------------------------------------------------------------|
|               |                                         |                                             | CBA/N Females                                                                 |
| I             | –                                       | –                                           | 1.14 ± 0.46 (14) 1.06 ± 0.21 (12)                                                |
| II            | 0                                       | –                                           | 3.36 ± 0.28 [100] 3.39 ± 0.17 [100]                                               |
| III           | 0                                       | −2                                          | 2.55 ± 0.24 [14]† 2.21 ± 0.42 [6]§                                                |
| IV            | 0                                       | +4                                          | 2.41 ± 0.23 [11]‡ 2.83 ± 0.14 [27]§                                               |
| V             | 0                                       | +20                                         | 3.40 ± 0.13 [109]|| 2.67 ± 0.49 [18]||                                               |
| VI            | 0                                       | +48                                         | 3.51 ± 0.12 [141] (2549) 3.25 ± 0.25 [73] (1818)                                  |

* Five mice per group; all injections were given i.p.
† Differs from group II; P < 0.05.
§ Differs from group II; P < 0.025.
|| Does not differ significantly from group II of the corresponding experiment.

### Table V

**Failure of Prior Immunization with DNP<sub>22</sub>-KLH to Prepare CBA/N × C3H/HeN F<sub>1</sub> Mice for Responsiveness to DNP<sub>59</sub>-Ficoll**

| Group number* and sex | First immunization given on day 0 | Second immunization given on day 15 | Mean log DNP-reactive PFC/spleen ± SE† (geometric mean) |
|-----------------------|-----------------------------------|-------------------------------------|--------------------------------------------------------|
|                       |                                   |                                    | Direct PFC                                                                 |
| I. Male               | –                                 | 10 µg DNP<sub>59</sub>-Ficoll      | 0.89 ± 0.36 (8) 1.13 ± 0.30 (14)                           |
| II. Male              | 50 µg DNP<sub>59</sub>-KLH        | –                                  | 2.56 ± 0.65 (367) 2.71 ± 0.68 (514)                       |
| III. Male             | 50 µg DNP<sub>59</sub>-KLH        | 10 µg DNP<sub>59</sub>-Ficoll      | 3.20 ± 0.13§ (1,007) 3.38 ± 0.09§ (2,448)                |
| IV. Male              | 50 µg DNP<sub>59</sub>-KLH        | 50 µg DNP<sub>59</sub>-KLH         | 3.81 ± 0.34 (6,572) 4.05 ± 0.29 (11,431)                |
| V. Female             | –                                 | 10 µg DNP<sub>59</sub>-Ficoll      | 4.86 ± 0.05 (73,958) 4.60 ± 0.05 (40,075)                |
| VI. Female            | 50 µg DNP<sub>59</sub>-KLH        | –                                  | 3.47 ± 0.06 (2,994) 3.28 ± 0.03 (1,936)                |
| VII. Female           | 50 µg DNP<sub>59</sub>-KLH        | 10 µg DNP<sub>59</sub>-Ficoll      | 4.37 ± 0.07§ (23,815) 4.23 ± 0.07§ (16,991)              |
| VIII. Female          | 50 µg DNP<sub>59</sub>-KLH        | 50 µg DNP<sub>59</sub>-KLH         | 4.27 ± 0.28 (18,675) 4.46 ± 0.31 (29,087)              |

* Five mice per group.
† All spleens assayed on day 19.
§ Does not differ significantly from group II; P > 0.10.
|| Differs from group V; P < 0.0025.
The Effects of Prior Immune Status on the Capacity to Respond to DNP_{59} Ficoll. Although CBA/N mice and the hybrid male progeny of CBA/N females are unable to respond directly to DNP_{59} Ficoll, the existence of a specific hapten-Ficoll-mediated blockade implies a significant interaction of their B cells with this polysaccharide agent. Conceivably, B cells from DNP_{22}-KLH-primed CBA/N mice might recognize the DNP_{59} Ficoll signal as immunogenic. Thus, priming with DNP_{22}-KLH might induce in defective mice some DNP-reactive memory B cells which could then undergo activation on subsequent encounter with DNP_{59} Ficoll.

Male and female hybrid mice were immunized with DNP_{22}-KLH and after 15 days, were challenged either with DNP_{22}-KLH or with DNP_{59} Ficoll. The results of splenic PFC assays performed 4 days later are presented in Table V. Memory to DNP_{22}-KLH had been generated in both the male and female hybrid mice since their indirect PFC already exceeded their direct PFC on the 4th day post challenge (groups IV and VIII). In hybrid female mice, challenge with DNP_{59} Ficoll caused both the direct and indirect PFC responses to be lower in DNP_{22}-KLH-primed than in unprimed animals (compare group VII with group V). It is important to note that for hybrid females, the immunogenicity of DNP_{59} Ficoll is not neutralized by any circulating antibodies produced in response to prior immunization with DNP_{22}-KLH (compare groups VI and VII).

In male hybrid mice, the 4th-day direct and indirect PFC responses after challenge with DNP_{59} Ficoll were both modestly elevated; but, the response levels did not differ significantly from the residual activity observed in control mice immunized 19 days earlier with DNP_{22}-KLH (compare group III with group II). It is conceivable that some defective F1 male memory cells may be ultimately triggered by subsequent contact with DNP_{59} Ficoll; however, the effect as currently observed falls far short of functional restoration.

Attempts to Impose Hapten-Specific Blockade on Memory Cell Populations in CBA/N Mice. Although generation of hapten-specific memory cell populations in defective mice conferred little if any capacity to respond directly to DNP_{59} Ficoll, it was important to ascertain whether memory B cells, like precursor B cells, were susceptible to haptenated-Ficoll blockade. CBA/N mice were immunized with DNP_{22}-KLH, and 10 days later they were challenged with the same antigen either with or without coexistent DNP_{59} Ficoll blockade. Our findings are recorded in Table VI. Again, indirect PFC outnumber direct PFC at 4 days after secondary challenge with DNP_{59} Ficoll (compare group III with group II). DNP_{59} Ficoll could not produce significant reductions in either the direct or indirect PFC responses of these memory cell-bearing defective CBA/N mice. Thus, the generation of memory cell populations allows escape from blockade in these defective mice even though it does not confer any capacity for direct responsiveness to DNP_{59} Ficoll. Whether or not circulating antibody elicited by prior encounter with DNP_{22}-KLH could neutralize the blockading capacity of DNP_{59} Ficoll is uncertain. Such antibody does not neutralize the immunogenicity of DNP_{59} Ficoll in normal hybrid female mice (Table V).

The Effect of DNP_{59} Ficoll-Mediated Blockade on the Development of B-Cell Memory to DNP_{22}-KLH. Although DNP_{59} Ficoll virtually abrogates the 4th-day secretory B-cell response to DNP_{22}-KLH, it was not clear whether DNP_{59}-
**TABLE VI**

Failure to Impose Blockade on Memory Cell Populations in CBA/N Mice

| Group number | Primary immunization | Blocking agent* | Secondary challenge‡ | Mean log DNP-reactive PFC/spleen ± SE$ (geometric mean) |
|--------------|----------------------|-----------------|----------------------|--------------------------------------------------------|
|              |                      |                 |                      | Direct | Indirect |
| I.           | DNP$_{22}$-KLH      | -               | -                    | 3.06 ± 0.11 | 2.37 ± 0.08 |
|              |                      |                 |                      | (1157) | (236) |
| II.          | -                    | -               | DNP$_{22}$-KLH       | 3.78 ± 0.12 | 2.94 ± 0.11 |
|              |                      |                 |                      | (8020) | (886) |
| III.         | DNP$_{22}$-KLH      | -               | DNP$_{22}$-KLH       | 3.32 ± 0.14 | 4.05 ± 0.03 |
|              |                      |                 |                      | (2082) | (11,112) |
| IV.          | DNP$_{22}$-KLH      | DNP$_{50}$-Ficoll | DNP$_{22}$-KLH       | 3.21 ± 0.16 | 3.82 ± 0.16 |
|              |                      |                 |                      | (1819) | (6,545) |

* 10 µg given 2 h before secondary challenge.
‡ 50 µg on day 10.
§ Assayed 4 days post secondary challenge. Five mice in each group.
¶ Does not differ from group III; $P \geq 0.10.$

Ficoll imposed an absolute blockade of all signals receivable by B cells from DNP$_{22}$-KLH. Accordingly, CBA/N mice were blockaded with DNP$_{50}$-Ficoll before an initial immunization with DNP$_{22}$-KLH, and 1 mo later they were challenged with DNP$_{22}$-KLH either with or without secondary blockade. Our findings are presented in Table VII. The defective CBA/N mice were blockaded on day 0 and challenged on day 30. They produced indirect PFC responses almost as high as those seen in control mice never subjected to blockade (compare groups III and IV). In addition, mice given a second blockade at the time of the 30 day challenge were now also completely refractory to blockade (groups III vs. V). Since we had previously shown that the effect of blockade is

---

**TABLE VII**

Failure of Blockade to Prevent Development of Memory Cell Populations in CBA/N Mice

| Group number | Primary blockade† | Primary immunization | Secondary blockade‡ | Secondary immunization | Mean log DNP-reactive PFC/spleen ± SE$ (geometric mean) |
|--------------|-------------------|----------------------|---------------------|------------------------|--------------------------------------------------------|
|              |                   |                      |                     |                        | Direct | Indirect |
| I.           | -                 | DNP$_{22}$-KLH       | -                   | -                      | 4.11 ± 0.13 | 2.86 ± 0.15 |
|              |                   |                      |                     |                        | (12,769) | (732) |
| II.          | DNP$_{50}$-Ficoll | DNP$_{22}$-KLH       | -                   | -                      | 4.18 ± 0.07 | 3.06 ± 0.08 |
|              |                   |                      |                     |                        | (1,067) | (1,150) |
| III          | -                 | DNP$_{22}$-KLH       | -                   | DNP$_{50}$-KLH         | 3.34 ± 0.06 | 4.43 ± 0.05 |
|              |                   |                      |                     |                        | (2,204) | (27,180) |
| IV.          | DNP$_{50}$-Ficoll | DNP$_{22}$-KLH       | -                   | DNP$_{50}$-KLH         | 2.73 ± 0.13 | 4.50 ± 0.12 |
|              |                   |                      |                     |                        | (538) | (19,820) |
| V.           | DNP$_{50}$-Ficoll | DNP$_{50}$-KLH       | DNP$_{50}$-Ficoll   | DNP$_{22}$-KLH         | 3.33 ± 0.22 | 4.36 ± 0.00 |
|              |                   |                      |                     |                        | (2,157) | (29,051) |

* Tested by challenge at 30 days post primary blockade and immunization. Five mice per group.
† 10 µg DNP$_{50}$-Ficoll given 1 h before immunization with 50 µg DNP$_{22}$-KLH.
‡ Assayed 4 days after challenge.
§ Does not differ significantly from group III; $P < 0.20.$
¶ Does not differ significantly from group IV; $P < 0.40.$
TABLE VIII
Failure of DNP₅⁹-Ficoll-Mediated Primary Blockade to Affect the Secondary Immune Response to DNP₂₂-KLH in CBA/N Mice*

| Group number | Primary blockade | Primary immunization§ | Secondary blockade | Secondary immunization¶ | Mean log DNP-reactive PFC/spleen = SE** (geometric mean) |
|--------------|------------------|------------------------|--------------------|--------------------------|----------------------------------------------------------|
|              |                  |                        | Direct PFC         | Indirect PFC             |
| I            | DNP₂₂-KLH        | DNP₂₂-KLH              | 3.75 ± 0.27        | 2.91 ± 0.11              | (5659) (821)                                             |
| II           | -                | DNP₅⁹-Ficoll           | 1.02 ± 0.44        | 0.47 ± 0.17              | (1) (3)                                                 |
| III          | DNP₅⁹-Ficoll     | DNP₅⁹-KLH              | 3.20 ± 0.19        | 3.78 ± 0.08              | (1599) (6601)                                           |
| IV           | DNP₅⁹-Ficoll     | DNP₅⁹-KLH              | 3.14 ± 0.19        | 3.82 ± 0.0341            | (1396) (6648)                                           |
| V            | DNP₅⁹-Ficoll     | DNP₅⁹-KLH              | 2.68 ± 0.11§       | 3.50 ± 0.16§             | (485) (3182)                                            |

* Tested by challenge at 8 days post primary blockade and immunization. Five mice per group.
† 10 μg DNP₅⁹-Ficoll given on day 0.
§ 50 μg DNP₅⁹-KLH on day 0 given 2 h after the DNP₅⁹-Ficoll blockade.
¶ 10 μg DNP₅⁹-Ficoll given on day 8.
§§ 50 μg DNP₅⁹-KLH on day 8 given 2 h after the second DNP₅⁹-Ficoll blockade.
** Assayed 4 days after challenge.
§ Does not differ significantly from group III; P < 0.40.
¶§ Differs from group IV; P < 0.05.
¶¶ Does not differ significantly from group IV; P < 0.10.

Discussion

The B Cell as the Probable Target of Hapten-Polysaccharide-Mediated Blockade. Our current findings focus attention on B cells as the probable direct targets of the X-linked hapten-specific blockade phenomenon. Polysaccharides carrying the tripeptide-enlarged DNP hapten were profoundly more efficient in producing blockade than was another polysaccharide bearing the traditional small form of the dnp hapten (Fig. 1). This implies a critical role for Ig receptors in the blockade phenomenon, as does the fact that increased haptenic density on the polysaccharide agents results in increased blocking efficiency. Multiple Ig receptors probably must be cross-linked by haptenated polymer for blockade to ensue, since unjoined free hapten and free Ficoll carrier (each provided at 100-fold their usual conjugated dosage) were unable to produce reduced with time after exposure to DNP₅⁹-Ficoll (Fig. 2), it was important to recheck these findings at a time when memory cell populations might reasonably be expected to first appear. The same experiment was repeated, but the DNP₂₂-KLH challenge was given on day 8 rather than on day 30. Our findings are presented in Table VIII. Prior blockade with DNP₅⁹-Ficoll did not prevent the full development of memory B-cell populations in response to the concomitant administration of DNP₂₂-KLH (groups III vs. IV). Efforts to produce secondary blockade at day 8 again failed to significantly reduce the indirect PFC response; the reduction of the direct PFC response, however, was marginally significant (groups IV vs. V). The overall effect stands in marked contrast to the effectiveness of DNP₅⁹-Ficoll in blockading the primary 4th-day secretory PFC response to DNP₂₂-KLH. Apparently, blockade of 4th-day secretory B-cell responses is of little predictive value for blockade of memory B-cell responsiveness in CBA/N mice.
Blockade. In addition, DNP₃₉-Ficoll given at the usual 10 μg dosage level could not be functionally displaced by prior injection of a 50-fold excess of nonhaptene-
ated AECM₁₁₂₀-Ficoll (Table III).

It has been proposed (6) that the CBA/N B-cell defect in responsiveness to thymic-independent antigens may be expressed at any of three levels: (a) a failure to bind thymic-independent antigens on B-cell surfaces, (b) a failure to recognize thymic-independent antigens once they are bound on B-cell surfaces or (c) the absence of a unique B-cell subpopulation with the capacity to respond to thymic-independent antigens. Our present findings indicate that CBA/N mice do have B cells which can recognize polysaccharide antigens; the recognition event, however, leads uniquely to B-cell blockade rather than to B-cell activation. Thus, it is still possible that CBA/N mice may be intrinsically unable to recognize haptene polysaccharides in any way that would lead to secretory activation of their defective B cells. In general, our present findings favor the view that in unprimed CBA/N mice, a unique B-cell subpopulation is functionally missing.

Possible Mechanisms of B-Cell Blockade. The presence of a B-cell defect in CBA/N mice is well established (3, 4). If indeed macrophages and T cells are not directly involved in the intrinsic defect, then hypotheses which emphasize B-cell characteristics may be constructed in an attempt to explain the functional defect. In principle, haptene-specific blockade of relevant B-cell clones could occur through critical interactions between Ig receptors on such B cells and (a) monovalent haptens, (b) the Ficoll carrier molecule (here a disparate or unique secondary receptor specific for polysaccharide would be required to initiate the blocking signal), (c) both of the above, i.e., separate recognition of both the haptene and the carrier moieties by the same cell, or (d) multivalent haptene. In this latter case, no receptor specialized for recognition of the polymeric carrier would be required, but a geometrically acceptable presentation of the haptenic epitopes to surface Ig receptors would suffice to effect blockade. In principle, this mechanism would allow blockade by haptene polymers other than haptene polysaccharides, as long as the critical requirements of epitope presentation geometry were met (22). Our current data appear to be supportive of either mechanism (c) or (d). If mechanism (d) is operative, then the requirements for critical epitope presentation can be about equally well met by either linear or multi-branched haptene polysaccharide blocking agents (Fig. 1).

The joint signal provided by haptene-conjugated carrier could be either passive or active in nature. It is conceivable that the multiple site interaction which occurs between Ig receptors and multivalent haptene Ficoll prevents disso-
ciation of haptene from these Ig receptors, and thereby assures saturative

---

2 Although current findings provide no support for these first two alternatives, final consider-
ation of these possibilities is being temporarily held in abeyance since higher test concentrations of free haptene and/or free carrier can be attained in in vitro or cell transfer systems.

3 Two sets of authors have recently reported the absence of unique differentiation antigens on spleen cells from CBA/N and/or hybrid male mice (25, 26). To the best of our knowledge, there are no reports claiming the presence of any additional unique antigens or receptors on lymphocytes from these B-cell defective mice.
preemption of most available receptors. Such a blockade mechanism would be passive in nature and would not necessarily require metabolic changes in the cell. At present, no adequate explanation can be offered for why cell capping and consequent shedding of antigen-antibody complexes would not provide for an early escape of B cells from such hapten-specific blockade.

The marked effectiveness of X-linked hapten-polysaccharide-mediated blockade, even at extremely low dosage levels (Fig. 1), seems consistent with an active role for haptenated polysaccharide blocking agents. In this instance, the joint signal provided by haptenated polysaccharide would produce distinct metabolic changes in the B cell. These changes would make the B cell refractory to subsequent thymic-dependent signals for secretory activation. Presumably refractoriness to secretory activation would not preclude memory cell activation.

A related concept has recently been proposed to account for B-cell activation in normal mice (22). According to this concept, an activation signal for normal B cells can be accomplished by the formation of "immunons" at B-cell surfaces. An "immunon" is formed when ≈12-16 Ig receptors are linked together by antigen in a spatially continuous cluster which can contribute to an activation signal. The number of geometrically stable "immunons" which must be formed on any given B-cell surface in order to cause its activation is unknown. Since many thymic-independent antigens are intrinsically nonmitogenic (5), and are thus presumably incapable of delivering an ordinary mitogenic second signal, the "immunon" concept is especially attractive.

The currently advanced alternative (which might apply only to defective CBA/N mice) requires only that a similar geometrically stable arrangement of cell surface Ig might be generated by haptenated polysaccharide agents. These blocking agents would then actively lead to a refractory B-cell state. For convenience, such a critical arrangement of cell surface Ig receptors might be termed a "toleron." Conceivably, the generally increased density of Ig surface receptors on the B cells of CBA/N mice (6) might predispose to "toleron" development.

Refractoriness of B Memory Cells to Hapten-Polysaccharide-Mediated Blockade. Although CBA/N mice appear to be delinquent in the development of mature IgM to IgD-type cell surface receptor relationships (7, 8, 23), prior immunization with a thymic-dependent antigen generates specific B memory cell populations. This maturational change is accompanied, no doubt, by marked changes in the representation and density of surface Ig receptors on the specifically affected B-cell clones (11, 12). Thus, some responding B cells may escape their usual prolonged infancy. These memory cells are apparently no longer susceptible to haptenated polysaccharide-induced blockade, but they have still not acquired any appreciable capacity for direct responsiveness to haptenated polysaccharide antigens (Table V). Why changes in Ig receptor density could lead to escape from susceptibility to blockade without recovery of some capacity for direct responses to polysaccharide antigens cannot be readily explained. One possibility is that circulating antibody generated at the time of memory cell induction with DNP₂⁻⁻KLH could effectively intercept and neutralize the blockading capacity of DNP₅⁻⁻Ficoll. In this case, the memory cells would not actually be subjected to blockade, and their failure to respond to
challenge with DNP_{59}-Ficoll would simply represent maintenance of their innate unresponsiveness to that antigen.

The early observations of Baker et al. (24) extended by Schrader and Nossal (13) have demonstrated that even "end-stage" secretory B cells are susceptible to external stimuli imposed by surface-bound antigens. This phenomenon has been termed effector cell blockade, and Schrader has recently shown (14) that it is accomplished by multipoint interactions with Ig receptors. He has also shown that specific enzymatic interruption of such Ig linkages results in the reversibility of the delivered signal. The work of Klaus has emphasized that B precursor cells of normal mice are also susceptible (at quite high dosages) to specific hapten-polysaccharide-imposed blockade (10-12). Thus, it seems reasonable to assume that B precursors as well as B secretory cells might well be susceptible to Ig receptor-mediated cell surface stimuli.

The fact that those conditions optimal for secretory B-cell blockade failed almost completely to cause B memory cell blockade indicates that some signal from the thymic-dependent DNP_{24}-KLH is reaching at least some B cells. It is difficult to imagine that such near total escape from blockade could occur if blockade is routinely accomplished by a metabolically active but reversible switching event on B precursor cells. This argument seems especially cogent since memory cell formation was virtually complete when tested at 8 days post-blockade (Table VIII), but secretory cell detection was still minimal when primary DNP_{24}-KLH stimulation was given as late as 14 days after DNP_{59}-Ficoll injection (Fig. 2). At present it is not possible to tell whether the precursors for secretory and for memory B cells represent two distinct subpopulations in CBA/N mice, or whether there is actually only one targeted precursor population. If a single precursor population is involved, then the signal requirements for immunogen-triggered differentiation into memory cells must be minimal as compared to those required for maturation into immediate secretory function. In any case, memory cell formation in these B-cell defective mice must clearly escape blockade more readily than secretory cell activation.

The Significance of Hapten-Polysaccharide-Mediated Selective Immune Blockade. The present study emphasizes the importance of Ig receptors for induction of specific hapten-polysaccharide-mediated B-cell blockade in defective CBA/N mice. It has disclosed the refractoriness of B memory cells to blockade, and the inevitability of memory cell development despite the presence of profound secretory cell blockade. It also raises many questions among which are: (a) is blockade strictly and exclusively a B-cell function? (b) does blockade represent merely a passive saturation of surface Ig receptors on B cells? (c) is participation of a second category of non-Ig receptors necessary for transmission of the blockade signal? (d) is the signal for blockade on any given B cell reversible? (e) can memory cell formation proceed in the presence of a complete and sustained secretory B-cell blockade? and finally, (f) to what extent do the cellular and molecular events which lead to the X-linked blockade of CBA/N B cells resemble the events which cause hapten-specific blockade of normal B cells? Clear answers to any of the first five questions will be distinctly useful, but a definitive answer to the final question could ultimately be of major importance in efforts to control detrimental B-cell responses.
Summary

CBA/N mice, a mutant CBA subline, harbor an X-linked B-cell defect which prevents them from mounting immune responses to certain thymic-independent antigens such as pneumococcal polysaccharides and haptenated-Ficoll derivatives. These mice and the hybrid male progeny of CBA/N females are also exquisitely sensitive to a hapten-specific blockade of their otherwise adequate immune responses to thymic-dependent antigens such as N-2,4-dinitrophenylated-hemocyanin (DNP-KLH). As little as 10 ng of a DNP-Ficoll conjugate given 2 h before immunization with a 5,000-fold greater dosage of DNP-KLH, virtually abolishes the 4th-day direct plaque-forming cell (PFC) response specific for DNP. Responding hybrid (CBA/N × C3H/HeN) female mice are resistant to such blockade even at DNP-Ficoll dosages increased by three orders of magnitude. The DNP hapten and Ficoll must be chemically joined for this blocking effect to occur, and increasing the hapten derivatization of Ficoll increases its blockade-invoking capacity. Significant blockade can be produced by administering DNP-Ficoll as early as 4 days before or as late as 4 h after immunization with DNP-KLH. All currently available data point to the defective B cell as the target of this hapten-polysaccharide mediated blockade. Mice bearing B memory cells, however, are refractory to such blockade. In addition, DNP-Ficoll injections which cause virtually total blockade of 4th-day primary direct PFC responses to DNP-KLH have little or no effect on the development of DNP-reactive B-cell memory measured at either 8 or 30 days. These findings suggest very different blockade susceptibilities for B cells or their precursors at various stages of differentiative development. Our findings also lead to the formulation of testable hypotheses regarding the mechanism of this selective B-cell blockade phenomenon.

We thank Barbara J. Duntley for her excellent technical assistance, and Dr. Phillip J. Baker for his helpful discussions and manuscript review.

Received for publication 28 February 1978.

References

1. Amsbaugh, D. F., C. T. Hansen, B. Prescott, P. W. Stashak, D. R. Barthold, and P. J. Baker. 1972. Genetic control of the antibody response to type III pneumococcal polysaccharide in mice. I. Evidence that an X-linked gene plays a decisive role in determining responsiveness. J. Exp. Med. 136:931.
2. Scher, I., M. M. Frantz, and A. D. Steinberg. 1973. The genetics of the immune response to a synthetic double-stranded RNA in a mutant CBA mouse strain. J. Immunol. 110:1396.
3. Scher, I., A. Ahmed, D. M. Strong, A. D. Steinberg, and W. E. Paul. 1975. X-linked B-lymphocyte immune defect in CBA/HN mice. I. Studies of the function and composition of spleen cells. J. Exp. Med. 141:788.
4. Scher, I., A. D. Steinberg, A. K. Berning, and W. E. Paul. 1975. X-linked B-lymphocyte immune defect in CBA/N mice. II. Studies of the mechanisms underlying the immune defect. J. Exp. Med. 142:657.
5. Mosier, D. E., I. Scher, H. Ruhl, P. L. Cohen, I. Zitron, and W. E. Paul. 1975. Activation of normal and defective B lymphocytes by thymus-independent antigens. In Role of Mitogens in Immunobiology. J. J. Oppenheim and D. Rosenstreich, editors. Academic Press, Inc., New York. 313.
6. Scher, I., A. Ahmed, S. O. Sharrow, A. D. Steinberg, and W. E. Paul. 1975. Genetic control of B-lymphocyte function in the CBA/N mouse strain: a model for examining the mechanism of B lymphocyte activation. In Role of Mitogens in Immunobiology. J. J. Oppenheim and D. Rosenstreich, editors. Academic Press, Inc., New York. 325.

7. Scher, I., S. O. Sharrow, and W. E. Paul. 1976. X-linked B-lymphocyte defect in CBA/N mice. III. Abnormal development of B-lymphocyte populations defined by their density of surface immunoglobulin. J. Exp. Med. 144:507.

8. Scher, I., S. O. Sharrow, R. Wistar, Jr., R. Asofsky, and W. E. Paul. 1976. B-lymphocyte heterogeneity: ontogenetic development and organ distribution of B-lymphocyte populations defined by their density of surface immunoglobulin. J. Exp. Med. 144:494.

9. Merchant, B., H. Snippe, E. F. Lizzio, and J. K. Inman. 1978. X-linked genetic control of hapten-polysaccharide-mediated specific immune unresponsiveness in CBA/N mice. J. Immunol. 120:1362.

10. Klaus, G. G. B., and J. H. Humphrey. 1975. B cell tolerance induced by polymeric antigens. I. Comparison of the dose and epitope density requirements for inactivation of primed and unprimed B cells in vivo. Eur. J. Immunol. 5:361.

11. Klaus, G. G. B. 1975. B cell tolerance induced by polymeric antigens. II. Effects of tolerance on hapten-binding lymphocyte levels in primary and secondary antibody responses. Eur. J. Immunol. 5:366.

12. Klaus, G. G. B. 1976. B cell tolerance induced by polymeric antigens. IV. Antigen-mediated inhibition of antibody-forming cells. Eur. J. Immunol. 6:200.

13. Schrader, J. W., and G. J. V. Nossal. 1974. Effector cell blockade. A new mechanism of immune hyporeactivity induced by multivalent antigens. J. Exp. Med. 138:1582.

14. Schrader, J. W. 1975. Effector cell blockade. II. A demonstration of the reversible masking of an immune response by blockade of antibody-forming cells. Eur. J. Immunol. 5:808.

15. Inman, J. K., B. Merchant, and S. E. Tacey. 1973. Synthesis of large haptenic compounds having a common functional group that permits covalent linkage to proteins, cell surfaces and adsorbents. Immunochemistry. 10:153.

16. Inman, J. K., B. Merchant, L. Claflin, and S. E. Tacey. 1973. Coupling of large haptenes to proteins and cell surfaces: preparation of stable, optimally sensitized erythrocytes for hapten-specific, hemolytic plaque assays. Immunochemistry. 10:165.

17. Inman, J. K. 1975. Thymus independent antigens: the preparation of covalent hapten-Ficoll conjugates. J. Immunol. 114:704.

18. McMaster, P. R. B., J. D. Owens and W. E. Vannier. 1977. The preparation and characterization of a thymic independent antigen: ε-Dinitrophenyl-L-lysine-Ficoll. Immunochemistry. 14:189.

19. Merchant, B., and B. Petersen. 1968. The sensitivities of two hemolytic plaque techniques as measures of cellular antibody production. J. Immunol. 101:860.

20. Desaymard, C., and H. Waldmann. 1976. Evidence for inactivation of precursor B cells in high dose unresponsiveness. Nature (Lond.). 264:780.

21. Desaymard, C., B. Pearce, and M. Feldmann. 1976. Role of epitope density in the induction of tolerance and immunity with thymus-independent antigens III. Interaction of epitope density and receptor avidity. Eur. J. Immunol. 6:546.

22. Dintzis, H. M., R. Z. Dintzis, and B. Vogelstein. 1976. Molecular determinants of immunogenicity: the immunon model of the immune response. Proc. Natl. Acad. Sci. U. S. A. 73:3671.

23. Finkelman, F. D., A. H. Smith, I. Scher, and W. E. Paul. 1975. Abnormal ratio of membrane immunoglobulin classes in mice with an X-linked B-lymphocyte defect. J. Exp. Med. 142:1316.

24. Baker, P. J., P. W. Stashak, D. F. Amabaugh, and B. Prescott. 1971. Characterization of the antibody response to type III pneumococcal polysaccharide at the cellular
level. II. Studies on the relative rate of antibody synthesis and release by antibody producing cells. *Immunology.* 20:481.

25. Ahmed, A., I. Scher, S. O. Sharrow, A. H. Smith, W. E. Paul, D. H. Sachs, and K. W. Sell. 1977. B-lymphocyte heterogeneity: development and characterization of an alloantiserum which distinguishes B-lymphocyte differentiation alloantigens. *J. Exp. Med.* 145:101.

26. Huber, B., R. K. Gershon, and H. Cantor. 1977. Identification of a B-cell surface structure involved in antigen-dependent triggering: absence of this structure on B-cells from CBA/N mutant mice. *J. Exp. Med.* 145:10.