

MURINE B-CELL SUBPOPULATIONS RESPONSIVE TO T-DEPENDENT AND T-INDEPENDENT ANTIGENS*

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The functional properties of T and B lymphocytes and the nature of their participation in the immune response have been elucidated to a large extent in recent years (1). Evidence of functional heterogeneity within the T-cell compartment has been garnered by utilizing surface markers which distinguish subsets of cells (2). Although B cells also carry distinctive surface markers, evidence of functional subpopulations in the B-cell compartment is scant. B-cell heterogeneity was initially suggested by Playfair and Purves (3), who reported a differential capacity of B cells from diverse anatomical regions to synergize with T cells. Additional support can be found in fractionation studies in which cells capable of responding to T-dependent and T-independent forms of hapten-carrier conjugates were separable by velocity sedimentation (4).

Our laboratory has been engaged in studies of the immune response to the azobenzene-*p*-arsonate epitope in mono-, bi-, and polyfunctional forms (5, 6). Previous efforts have utilized the guinea pig as the laboratory model, since guinea pigs make vigorous cellular immune responses to small mono- and bifunctional azobenzene-*p*-arsonate (ABA)¹ derivatives. The present communication describes the initial phases of an investigation of the murine response to polyfunctional ABA conjugates. The findings indicate that strain A/J mice make plaque-forming cell (PFC) responses to the ABA epitope only when it is presented in a T-dependent form, and that the C3 receptor is a functional marker for murine B cells which respond to many T-dependent antigens (hapten-protein conjugates). Further, C3 may be an obligatory component in the pathway that leads to these T-dependent antibody responses, but the C3 receptor is apparently unexpressed on the surfaces of B cells which make T-independ-

* Supported by U. S. Public Health Service grants AI-05664 and AI-11983 and National Science Foundation grant GB-27591.

¹ Abbreviations used in this paper: ABA, azobenzene-*p*-arsonate; ABA-Ficoll, *m*-azobenzene-*p*'-arsonate-3-*p*-hydroxyphenyl-propionyl-aminoethyl-carbamyl methyl-Ficoll; AECM-Ficoll, aminoethyl-carbamyl methyl-Ficoll; BGG, bovine IgG; CFA, complete Freund's adjuvant; Con A, concanavalin A; CoV, cobra venom factor; CRL, complement receptor lymphocyte; DNP-Ficoll, 2,4-dinitrophenyl-aminoethyl-carbamyl methyl-Ficoll; DRAT, *m*-azobenzene-*p*'-arsonate-3-*p*-hydroxyphenyl-propionic acid; EAC, sheep erythrocytes sensitized with anti-sheep erythrocyte antibody and then sensitized with mouse C3; GVB, veronal buffer, pH 7.2, containing 0.1% gelatin, Ca⁺⁺ Mg⁺⁺; KLH, giant keyhole limpet hemocyanin; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; PFC, plaque-forming cell; RAT, L-tyrosine azobenzene-*p*-arsonate; RFC, rosette-forming cell.

ent antibody responses. These findings are in general accord with recent reports from other laboratories (7, 8).

Materials and Methods

Antigens and Immunization. Giant keyhole limpet hemocyanin (KLH) and bovine IgG (BGG) were obtained from Calbiochem, Los Angeles, Calif., and Miles Laboratories, Inc., Miles Research Div., Kankakee, Ill., respectively. Ficoll was obtained from Pharmacia Fine Chemicals, Inc., Piscataway, N. J. *Escherichia coli* lipopolysaccharide (LPS) K235 was supplied by Abbot Laboratories, Chicago, Ill. Concanavalin A (Con A) was purchased from Miles Laboratories, Inc., Miles Research Div. All other reagents were purchased from Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N. Y.

Conjugation of ABA was performed according to the method of Tabachnick and Sobotka (9), while 2,4-dinitrophenyl (DNP)-protein conjugates were prepared as described by Eisen (10). 2,4-Dinitrophenyl-aminoethyl-carbamyl methyl (DNP₈₇)-Ficoll and aminoethyl-carbamyl methyl (AECM₁₅₀)-Ficoll were prepared according to the method used by Inman (11). A mono-substituted conjugate of *p*-hydroxyphenyl-propionic acid (DRAT) was synthesized as described in an earlier publication (5). DRAT was coupled to AECM₁₅₀-Ficoll by generating the *N*-hydroxysuccinimide ester of DRAT with dicyclohexylcarbodiimide, and reacting the active ester with AECM₁₅₀-Ficoll in aqueous dimethylformamide. The resulting *m*-azobenzene-*p*'-arsonate-3-*p*-hydroxyphenyl-propionyl-aminoethyl-carbamyl methyl-Ficoll (ABA-Ficoll) was purified by dialysis and chromatography.

Female A/J and C57BL/6 mice 6-8 wk of age were purchased from The Jackson Laboratory, Bar Harbor, Maine and from Simonsen Laboratories, Gilroy, Calif., respectively.

For primary immunization, 50 µg ABA-KLH or 250 µg DNP-BGG emulsified in complete Freund's adjuvant (Difco Laboratories, Madison, Wis.), was administered intraperitoneally. Secondary immunization with ABA-KLH consisted of a single intraperitoneal injection of 50 µg of ABA-KLH dissolved in saline. All other antigens were administered intravenously in saline.

Plaque Assay. The technique of Cunningham and Szenberg (12) as described by Jerne et al. (13) was employed to detect the presence of antibody-forming cells. ABA-modified sheep erythrocytes (SRBC) were prepared at 0°C by reacting 0.12 ml of 0.111 M diazotized arsanilic acid with 10 ml of a thrice washed 5% SRBC suspension in phosphate-buffered saline (PBS), pH 7.4. The reaction was terminated after 10 min by washing with cold PBS. 2,4,6-trinitrophenyl-modified indicator cells were prepared by adding 2 ml of a 0.5% (wt/vol) solution of 2,4,6-trinitrobenzene sulfonic acid to 3 ml of PBS containing 0.5 ml of thrice washed SRBC. The reaction was allowed to proceed for 10 min at bench temperature followed by extensive washings with PBS and finally with plating medium.

Rosette Assay. The hapten-specific rosette assay is described in detail elsewhere (14). Briefly, single spleen cell suspensions were prepared with PBS containing sodium azide (1,000 µg/ml) at a final concentration of 4×10^7 viable cells/ml. One volume of spleen cells was mixed with one volume of PBS or PBS containing 5×10^{-4} M L-tyrosine ABA (RAT) and allowed to stand at ambient temperature for 30 min in order to facilitate receptor equilibration. An equal volume of 2% ABA-SRBC was added, the preparation chilled to 0°C, and centrifuged at 50 *g* for 10 min in a refrigerated centrifuge. The pelleted preparations were allowed to stand for 30 min at 0°C, followed by resuspension in the cold on a rotator for 10 min at 10 rpm/min. Rosettes were enumerated in counting chambers constructed from glass slides and cover slips using double stick tape. A minimum of 100 rosettes were scanned for each point.

C3 Depletion. Transient C3 depletion was achieved according to the method of Pepys (7). Each mouse received four intraperitoneal injections of cobra venom factor (CoV) (Cordis Laboratories, Miami, Fla.) at 8-h intervals. The dose ratio for CoV treatment was 200 U/kg body weight (i.e., approximately 4 units/mouse). Animals were immunized immediately after the last injection of CoV and assays for direct and indirect PFC were performed 7 days later.

Detection and Depletion of Complement Receptor Lymphocytes (CRL). Unpooled SRBC (Colorado Serum Co., Denver, Colo.) were washed three times and sensitized with a subagglutinating concentration of 19S rabbit anti-SRBC in veronal buffer, pH 7.2, containing 0.1% gelatin, Ca⁺⁺ Mg⁺⁺ (GVB). Sensitization was allowed to proceed at 37°C for 30 min and was terminated by washing three times with GVB containing 0.01 M EDTA, followed by two additional washes in

GVB. The sensitized SRBC were reacted for 30 min at 37°C with a 1:4 dilution of C5-deficient mouse serum. The resulting SRBC sensitized with rabbit anti-SRBC antibody and then sensitized with mouse C3 (EAC) were washed once with GVB-EDTA and twice with GVB.

Spleen cell suspensions were prepared as described above, freed of erythrocytes by lysis with NH_4Cl , and washed into RPMI-1640 medium containing 10% heat-inactivated fetal calf serum. EAC rosettes were formed by mixing 5×10^7 spleen cells with 1×10^6 EAC in 4 ml total volume. The mixture was rotated at bench temperature for 30 min followed by centrifugation. The supernate was removed and the cells resuspended in 2 ml of fresh medium by rotation. The fraction of rosetting cells was determined immediately in chambers described for the hapten-specific rosette assay. A minimum of 100 rosettes were scored for each assay.

Populations of spleen cells enriched for B cells were prepared by treatment with anti-Thy-1 serum (15) or by eluting the adherent cell population from a nylon wool column (16). The anti-Thy-1 serum was cytotoxic for about 40% of mouse spleen cells and 100% of thymic lymphocytes. EAC were formed and the rosetted [CRL(+)] and nonrosetted [CRL(-)] cells were separated (17) and treated with isotonic NH_4Cl to lyse RBC and washed in preparation for the Mishell-Dutton assay.

Cell Cultures. Fractionated splenic B cells were cultured (18) in medium containing 5×10^{-5} M 2-mercaptoethanol.

Mitogen-Induced Proliferation. Mitogens were dissolved or suspended in RPMI-1640 and 0.1 ml was added to each culture. Control cultures received 0.1 ml RPMI-1640. Preliminary studies established 0.5 μg as the optimal concentrations for LPS and Con A, respectively. Five hundred thousand lymphocytes suspended in 0.5 ml of RPMI-1640 containing 5% fetal calf serum (Grand Island Biological Co., Berkeley, Calif.) were cultured with or without mitogen for 3 days. On the last day of culture, 0.2 μCi of [methyl- ^{14}C]thymidine in 0.5 ml of RPMI-1640 was added. Proliferation was terminated by washing the cultures onto glass fiber filters followed successively by TCA and methanol washes. After drying, incorporated radioactivity was determined by liquid scintillation spectrometry.

Results

PFC Response of A/J Mice to ABA on a Thymic-Dependent Carrier. Murine anti-ABA PFC have been difficult to demonstrate (19), probably owing to the relatively rigid antigen dosage and immunization schedule found to be essential in this laboratory. In our experiments, primary responses were never seen, but priming with 50 μg of ABA-KLH in complete Freund's adjuvant (CFA) followed 5 wk later by a booster injection of 50 μg of the conjugate in saline consistently yielded significant indirect PFC responses on the 4th day after boosting ($111,000 \pm 8,000$ /spleen; Fig. 1). Priming with carrier alone was insufficient to induce an anti-ABA PFC response to a secondary injection of ABA-KLH (Fig. 1). No direct plaques were recorded after either the priming or boosting injections of ABA-KLH. The secondary PFC response is of very limited duration, as background levels were observed again by day 9 (Fig. 1). Although priming with ABA-KLH did not induce a PFC response, an increase in spleen cells which formed rosettes with ABA-SRBC occurred. After priming, the rosette-forming cell (RFC) level rose from a background of $1.1/10^4$ spleen cells to $5/10^4$ cells. The formation of rosettes was inhibitable by RAT.

PFC Response of A/J Mice to ABA on a Thymic-Independent Carrier. Since hapten-Ficoll conjugates have been reported to generate T-independent IgG as well as IgM antibody production (20), Ficoll was selected as a promising carrier to test the ability of A/J mice to respond to the ABA determinant in a thymic-independent form. Accordingly, ABA was linked to AECM-Ficoll via an active ester of DRAT and the resulting conjugate, ABA-Ficoll, as well as DNP-Ficoll, were employed as immunogens. AECM-Ficoll proved to be a suitable carrier for the DNP haptenic determinant, as anti-DNP direct PFC responses in the range

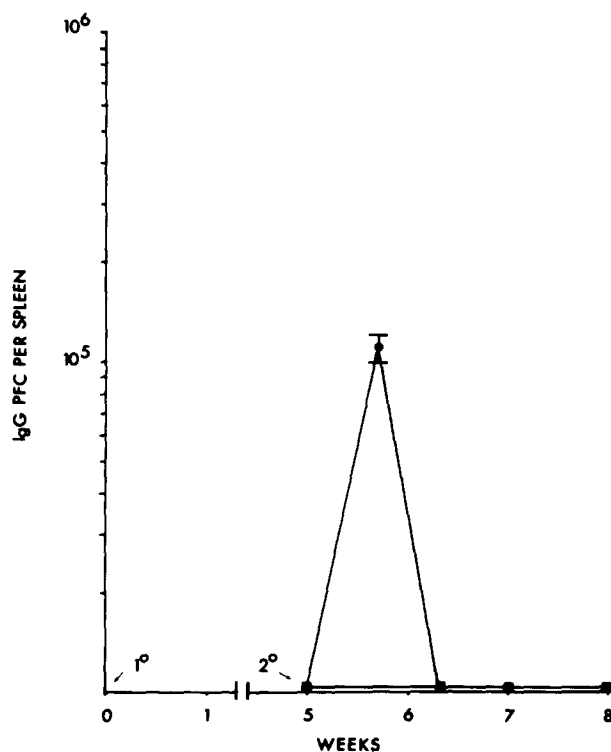


FIG. 1. Secondary indirect PFC response to ABA-KLH in A/J mice primed with either 50 μg KLH (squares) or 50 μg ABA-KLH (circles) in CFA and boosted with 50 μg ABA-KLH in saline 5 wk postpriming.

of $4-8 \times 10^4$ per spleen were obtained with an antigen dose range of 1-100 μg (Fig. 2). Although only direct PFC responses are shown, about 10^4 indirect anti-DNP PFC per spleen were observed by day 7. In contrast, ABA-Ficoll was unable to induce a perceptible anti-ABA PFC response over a similar time and dose range. As few as 10^3 PFC per spleen could readily have been detected by the assay. Although only responses on days 4 and 5 are illustrated in Fig. 2, negative results for anti-ABA direct and indirect PFC were obtained up to 28 days postimmunization. In addition to the absence of a PFC response to ABA-Ficoll, the level of detectable anti-ABA RFC declined after immunization from a background of $1.1/10^4$ to $0.5/10^5$ spleen cells.

Inability of ABA-KLH to Expand an ABA Precursor Population Responsive to ABA-Ficoll. The absence of a detectable primary response to ABA-KLH and the requirement for priming with ABA-KLH, as opposed to KLH alone, suggests that the frequency of ABA-specific B-cell precursors in the A/J mouse may be low. If B cells responsive to ABA-KLH can be triggered by ABA coupled to T-independent carriers, then it might be expected that priming with ABA-KLH and boosting with ABA-Ficoll would produce a significant anti-ABA response, as occurs when boosting with the homologous immunogen. Groups of three A/J mice were primed with 50 μg of ABA-KLH and boosted 5 wk later with 10, 100, or 1,000 μg of ABA-Ficoll or with 50 μg of ABA-KLH. The animals primed and

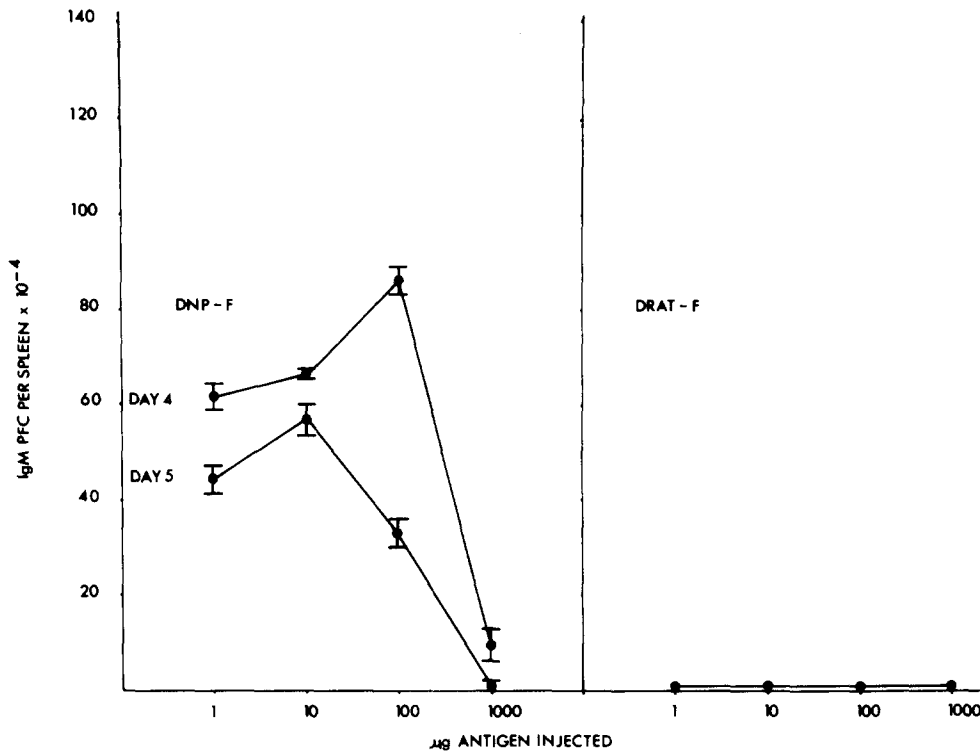


FIG. 2. Primary direct PFC response to DNP-Ficoll (F) and ABA-Ficoll in A/J mice. DNP-Ficoll (○) day 4, and (●) day 5. ABA-Ficoll (■) days 4 and 5. Read ABA for DRAT.

boosted with ABA-KLH had a mean of $31,107 \pm 2,202$ indirect PFC per spleen, whereas all groups of mice boosted with ABA-Ficoll were negative (Table I). These results suggest that ABA-Ficoll is incapable of triggering memory B cells generated by ABA-KLH.

Ability of ABA-Ficoll to Abrogate the anti-ABA Response to ABA-KLH. In order to exclude the inability of ABA-Ficoll to reach immunocompetent cells as an explanation for its apparent nonimmunogenicity, experiments designed to assess its tolerogenicity were carried out. Virgin A/J mice or animals which had been primed with ABA-KLH were given three injections of $200 \mu\text{g}$ ABA-Ficoll at 24-h intervals; immediately after the last injection, the mice were either primed or boosted with $50 \mu\text{g}$ ABA-KLH. Indirect PFC were determined 5 days after boosting. Administration of ABA-Ficoll before either priming or boosting with ABA-KLH reduced the secondary indirect PFC response by approximately 90% (Table II). In contrast, the anti-DNP PFC response to DNP-KLH was unaffected by identical treatment with ABA-Ficoll. These results indicate that ABA-Ficoll does reach immunocompetent spleen cells capable of responding to the ABA epitope. They are consistent with the observation that detectable RFC for ABA-SRBC decline after injection of ABA-Ficoll, but the relationship of RFC to precursors of PFC is unknown.

Effect of C3 Depletion on Anti-Hapten T-Dependent and T-Independent Responses. The foregoing results suggest the possibility that different subpopula-

TABLE I
Inability of ABA-KLH to Expand an ABA-Specific B-Cell Precursor Population Responsive to ABA-Ficoll

Group*	Priming injection	Boosting injection‡	PFC/spleen§
I	50 µg ABA-KLH	50 µg ABA-KLH	31,107 ± 2,202
II	50 µg ABA-KLH	10 µg ABA-Ficoll	<1,000
III	50 µg ABA-KLH	100 µg ABA-Ficoll	<1,000
IV	50 µg ABA-KLH	1000 µg ABA-Ficoll	<1,000

* Three A/J mice per group.

‡ 5 wk after priming injection.

§ Assayed 4 days after boosting injection.

TABLE II
Ability of ABA-Ficoll to Abrogate the Anti-ABA Response to ABA-KLH in A/J Mice

Group*	ABA-Ficoll‡	ABA-KLH → 5 wk →	ABA-Ficoll	ABA-KLH	PFC/spleen
I	—	50 µg	—	50 µg	45,238 ± 3,485
II	3 × 200 µg	50 µg	—	50 µg	5,249 ± 2,601
III	—	50 µg	3 × 200 µg	50 µg	4,666 ± 577

* Four mice per group.

‡ Administered at 24-h intervals in saline intraperitoneally.

tions of B lymphocytes may be involved in antibody responses to T-dependent and T-independent antigens. According to this scenario, anti-DNP precursors would reside in both B-cell compartments of A/J mice, based on anti-DNP PFC responses to DNP conjugates of T-dependent and T-independent carriers, whereas anti-ABA precursors would reside largely or exclusively in the T-dependent compartment. In order to critically evaluate this hypothesis, a marker other than antigen responsiveness capable of discriminating between B cells was required. Pepys has reported a differential effect of C3 depletion by CoV on murine responses to T-dependent and T-independent antigens (7). The antigens which he used were SRBC and polyvinylpyrrolidone, and it is difficult to assess to what degree epitope differences between the two might have been responsible for the effect observed. In addition, only IgM responses to the T-independent antigen were reported, leaving the possibility that C3 may be essential for IgG responses to T-independent as well as to T-dependent antigens.

In order to circumvent these ambiguities, responses to the same epitope (DNP) on a T-dependent carrier (BGG) and on a T-independent carrier (Ficoll) were assayed in C57BL/6 mice, which were used because they make vigorous T-independent IgG as well as IgM responses to DNP-Ficoll (20). However, parenthetically, we subsequently found that A/J mice make comparable IgG PFC responses to DNP-Ficoll. Both the IgM and IgG PFC responses to DNP-BGG were suppressed by approximately 90% in mice depleted of C3 as described by Pepys, whereas the IgM and IgG responses to DNP-Ficoll were unaffected by prior C3 depletion (Table III). These clear-cut results are similar to those of Pepys (7) and suggest the involvement of C3, or a product of activated C3, in T-dependent antibody responses but not in T-independent responses. They further show that T-independent IgG as well as IgM responses are refractory to C3 depletion.

TABLE III
The Effect of C3 Depletion by CoV on PFC Responses to DNP-BGG and DNP-Ficoll in C57BL/6 Mice

CoV treatment	Antigen	Direct PFC/spleen	Reduction	Indirect PFC/spleen	Reduction
			%		%
-	DNP-BGG	78,776 ± 16,512		109,736 ± 19,608	
+	DNP-BGG	7,560 ± 3,780	90	12,180 ± 1,260	89
-	DNP-Ficoll	87,837 ± 15,678		79,998 ± 9,648	
+	DNP-Ficoll	210,745 ± 23,872	0	169,715 ± 27,602	0

The effect of C3 depletion on hapten-specific memory was investigated by treating groups of A/J mice with CoV before priming, before boosting, or before both priming and boosting with ABA-KLH. C3 depletion before priming caused a moderate depression of the anti-ABA PFC response (ca. 50%), as did CoV treatment before priming and boosting (Fig. 3). However, the most marked effect (75% depression) was observed in mice treated only before the booster injection of antigen. Since depletion of C3 is transient, lasting about 4 days (6, 21), it is not surprising that the response was only moderately depressed in animals depleted only before priming. Sufficient antigen probably remained after C3 recovery to prime to some degree. The relative ineffectiveness of CoV administered before both priming and boosting may be attributable to its immunogenicity (6, 21), since neutralizing antibody would attenuate the effect of the dose given before the second injection of antigen. The observation that the anti-ABA PFC response was depressed approximately 75% when CoV was administered only before boosting indicates that the triggering of memory cells, as well as their induction, is dependent on the presence of C3.

In Vitro Response of Splenic CRL(+) and CRL(-) B Cells to a T-Independent Antigen. The insensitivity of the in vivo anti-DNP-Ficoll response to C3 depletion by CoV could be due to an absence of C3 receptors on the responding cell population, or, alternatively, receptors might be present but not required for the response to T-independent antigens. In order to distinguish between these alternatives, spleen cells from C57BL/6 mice were fractionated first into an enriched B-cell population by recovery from nylon wool or treatment with anti-Thy-1 serum, and then the B-cell fraction was resolved into CRL(+) and CRL(-) cells by EAC rosette formation. The CRL(+) cells comprised about 30% of the total spleen cell population. The subfractions as well as unrosetted B cells were cultured in vitro for 4 days at a density of 5×10^6 cells/ml. Cultures received either saline or 10 ng of DNP-Ficoll, a quantity determined by preliminary experiments to be the optimal immunogenic dose for splenic B cells.

The CRL(-) fraction made the best PFC response to DNP-Ficoll, averaging 238 ± 42 PFC per culture (Fig. 4). The response of the mixed B-cell population was substantially lower (164 ± 17 PFC per culture), the difference between the two groups being statistically significant ($0.05 > p$). The response of the CRL(+) fraction was the lowest of the three (37 ± 20 PFC per culture). The difference between the responses of the CRL(+) and CRL(-) populations was highly significant ($0.001 > p$). Only direct PFC were detected in these experiments,

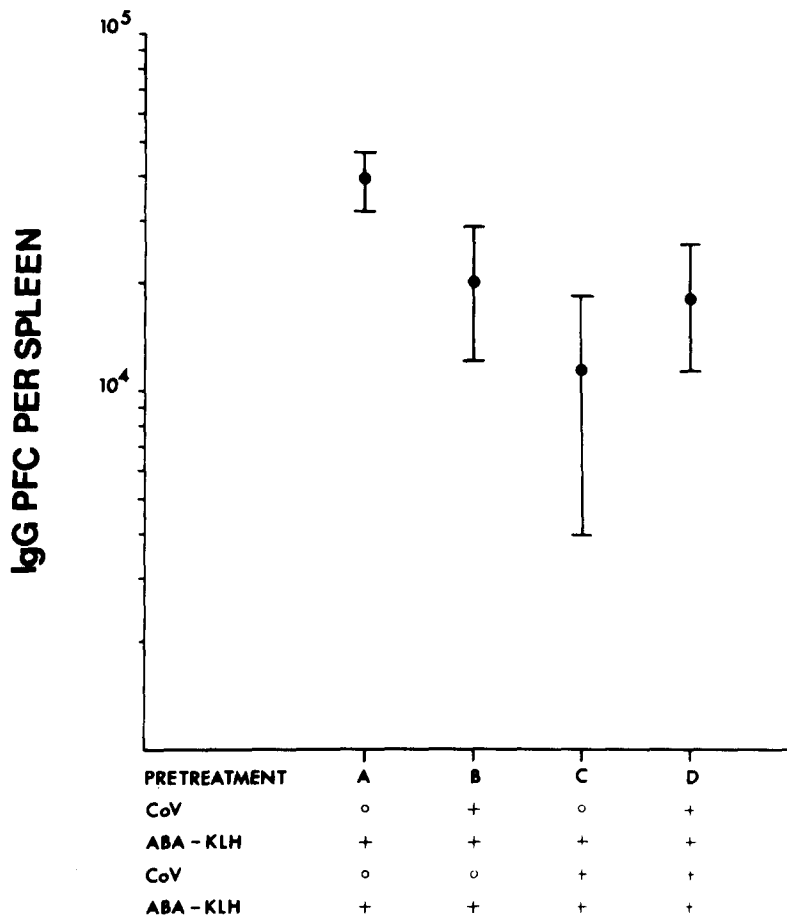


FIG. 3. Effect of CoV treatment on generation and elicitation of anti-ABA memory in A/J mice. C3 depletion was implemented immediately before immunization with ABA-KLH. The interval between priming and boosting was 5 wk. Indirect PFC were assayed 5 days after the booster injection of antigen.

owing perhaps to the reduced cell density used in the cultures, which was necessitated by the small yield of CRL(-) cells.

The results of these experiments indicate that the splenic B-cell population responsive to DNP-Ficoll, and presumably to T-independent antigens in general, does not bear a receptor for C3. They also strengthen the suggestion from the *in vivo* C3-depletion experiments that distinct subpopulations of B cells respond to T-dependent and T-independent antigens.

In Vitro Response of Splenic CRL(+) and CRL(-) B Cells to a T-Dependent Antigen. The preceding experiments with DNP-Ficoll established that the response to that antigen is essentially restricted to CRL(-) B cells, but did not exclude the possibility that CRL(-) cells might also cooperate with T cells in T-dependent antibody responses. In order to investigate this further, CRL(+) and CRL(-) B cells were assayed for PFC responses to a T-dependent antigen *in vitro*. Since it had been reported that substantial levels of anti-SRBC antibody

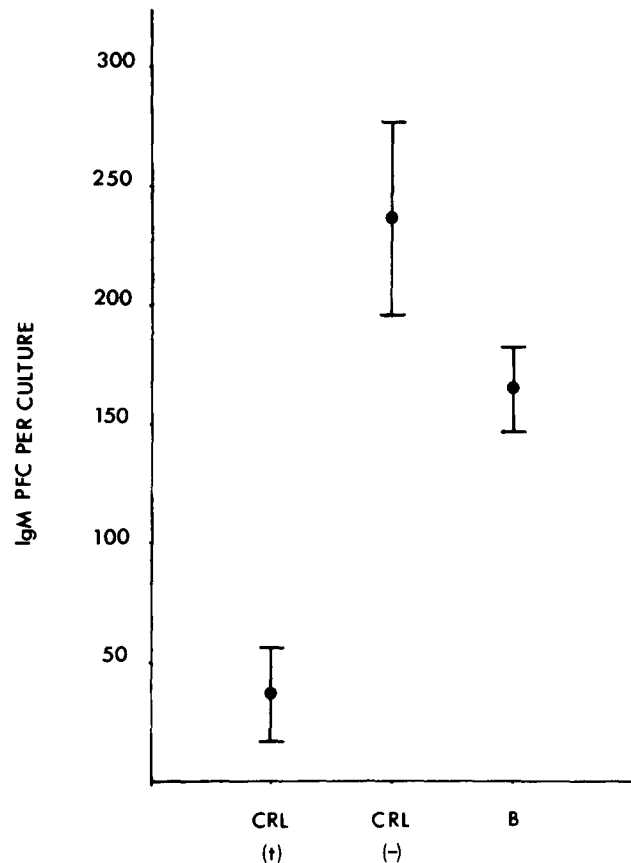


FIG. 4. Response of splenic B-cell populations from C57BL/6 mice to DNP-Ficoll. B cells were prepared from whole spleens using nylon wool and further fractionated on the basis of the C3 receptor. Direct PFC were assayed on day 4 after culture initiation.

were detectable in CoV-treated mice, though less than in untreated animals (21), suggesting the possibility of CRL(-) B-cell responses to this antigen, SRBC was selected as the T-dependent antigen in the present study.

Spleen cells from C57BL/6 mice primed 4 days previously with 4×10^8 SRBC per mouse were fractionated into CRL(+) and CRL(-) populations by EAC rosetting. The CRL(+) population was assumed to consist exclusively of B cells, which was supported by the inability of 10^7 CRL(+) cells to respond to SRBC in vitro (data not shown). On the other hand, the CRL(-) fraction contained primed T cells and accessory cells in addition to CRL(-) B cells. No rosettes were observed after scanning 3×10^3 cells after rosetting of the CRL(-) fraction; hence, contamination with CRL(+) cells was much less than 1%.

Various percentages of CRL(+) and CRL(-) cells were cultured with 3×10^6 SRBC for 5 days, at which time the cultures were assayed for IgM PFC. Cultures of unfractionated spleen cells in the absence of SRBC provided a background of approximately 100 PFC per culture (Fig. 5). Titration of CRL(+) cells by addition to the CRL(-) fraction yielded a linear cell dose-PFC response between 0 and 30% CRL(+) cells with a slope of approximately 3,100 IgM PFC per 10^6 CRL(+) cells (Fig. 5). The response plateaued between 30 and 50%, suggesting

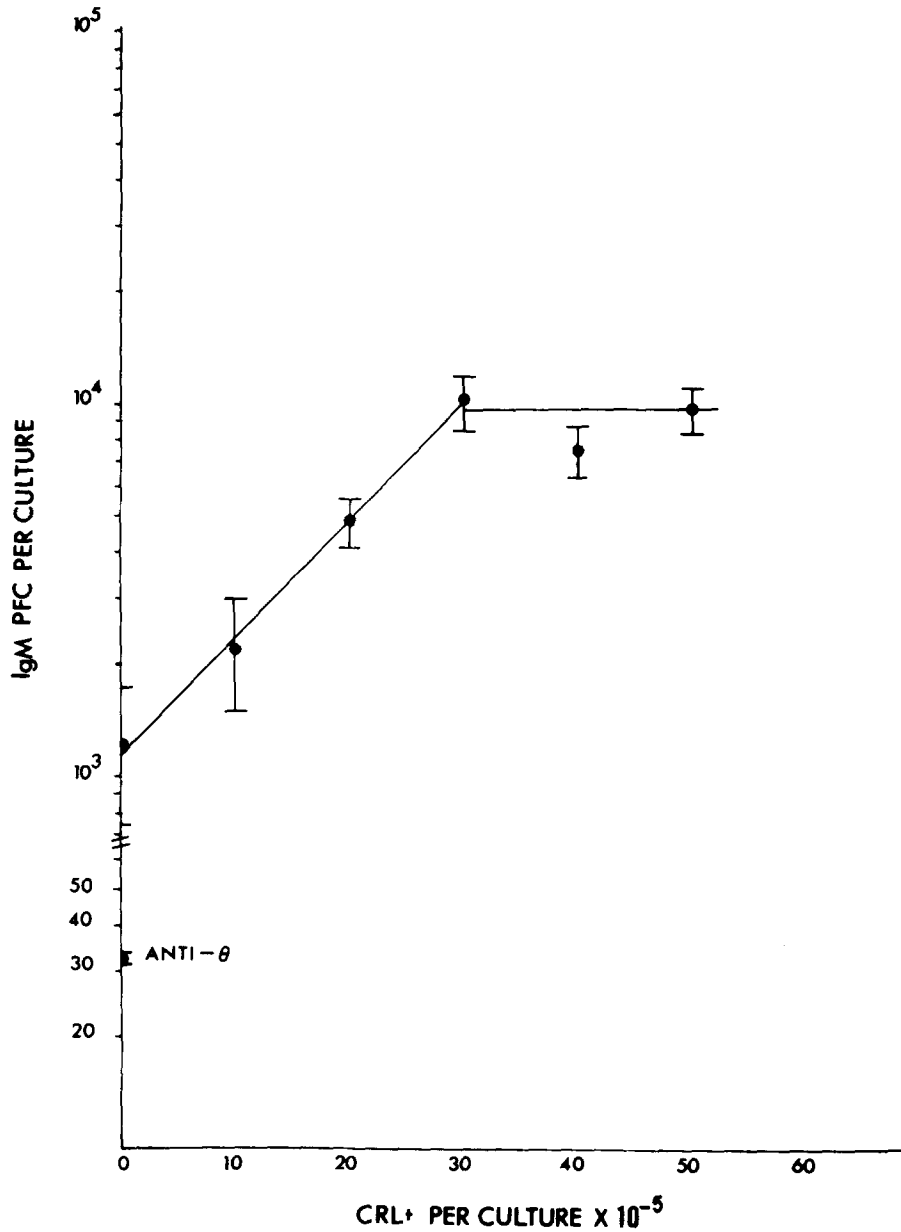


FIG. 5. Response of splenic B-cell populations from C57BL/6 mice to SRBC. CRL(+) B cells were prepared by formation of EAC rosettes. Cultures contained 10^7 cells comprised of varying proportions of CRL(+) and CRL(-) fractions. The T-cell dependence of the CRL(-) PFC response was assessed by treatment with anti-Thy-1 serum and complement before culturing. Direct PFC were assayed on day 5 after culture initiation.

that CRL(+) cells were no longer limiting. The normal frequency of CRL(+) cells in the spleen is about 31%. At 100% CRL(+) cells, the anti-SRBC response was not significantly different from background, as expected in the absence of T cells and macrophages.

Of particular interest, cultures to which no CRL(+) cells were added produced small but significant PFC responses ($1,754 \pm 564$ per culture). These responses were entirely abrogated by treating the cells with anti-Thy-1 serum and complement before culture (32 PFC per culture). In contrast, anti-Thy-1 treatment had no effect on the PFC response of CRL(-) cells to DNP-Ficoll (see above). Since contamination of the CRL(-) population by CRL(+) cells could account for no more than about 75 PFC per culture, which would not be significantly different from background, the results suggest that at least a small subpopulation of CRL(-) B cells make T-dependent antibody responses.

Response of CRL(+) and CRL(-) Cells to a Polyclonal B-Cell Mitogen. The CRL(+) and CRL(-) B-cell fractions were tested for their capacity to make proliferative responses to bacterial LPS, an established polyclonal mitogen for B cells. Both cell types made vigorous responses, although the CRL(-) cells were significantly more responsive in these assays than CRL(+) cells (Table IV). Neither population responded to Con A.

Discussion

The findings reported here indicate that B cells in A/J mice specific for the ABA epitope reside largely or exclusively in a subpopulation which requires T-cell cooperation, whereas B cells directed against the DNP determinant are found in T-independent as well as T-dependent compartments. The two subpopulations can be distinguished by the cell surface receptor for C3, which is present on T-dependent B cells but absent on those which respond to T-independent antigens. The evidence supporting this conclusion derives from the C3 depletion experiments *in vivo* reported here and elsewhere (7), in which antibody responses to ABA and DNP epitopes on T-dependent carriers were largely abrogated by CoV, whereas the PFC response to DNP on a T-independent carrier was unaffected. More definitively, the demonstration that the response to DNP-ficoll could be enriched in the CRL(-) population of B cells, prepared by EAC rosette fractionation, established not only that C3 did not participate in the response, but that precursor cells specific for this T-independent antigen did not, in fact, express C3 receptors. The C3 receptor, therefore, can be considered a marker for B cells which respond to T-dependent antigens.

Bitter-Suerman and colleagues have postulated that T-independent antigens activate B lymphocytes on the basis of their capacity to trigger the alternate complement pathway (22). While many T-independent antigens such as LPS possess this capacity as well as polyclonal mitogenicity, Ficoll neither activates the alternate pathway nor has appreciable mitogenic activity (23), findings which have been confirmed by us (unpublished observations). The two types of antigens are further distinguished by the cells on which they act; LPS activates CRL(+) as well as CRL(-) B cells, whereas Ficoll stimulates only the latter population. CRL(+) anti-ABA memory cells generated by priming with ABA-KLH could not be triggered by ABA-Ficoll. Experiments to determine if such memory cells can be triggered by ABA-LPS conjugates are in progress.

The anti-ABA PFC response in A/J mice has several curious features. In addition to the absence of a response to ABA-Ficoll, the response to ABA-KLH requires haptens as well as carrier priming, is essentially a pure IgG response,

TABLE IV
Responses of CRL(+) and CRL(-) B Cells to Polyclonal Mitogens

Cell type	Mitogen	
	LPS*	Con A*
Splenic B cells	4.17 ± 0.29‡	≤1
CRL(+) B cells	9.71 ± 2.95	≤1
CRL(-) B cells	26.20 ± 4.33	≤1

* 5×10^6 cells cultured with 0.5–5 μg LPS or 1–2 μg Con A.

‡ Stimulation index = (cpm ^{14}C -thymidine incorporated in cultures containing mitogen)/(cpm ^{14}C -thymidine incorporated in cultures without mitogen).

and exhibits a critical temporal relationship between priming and boosting. In order to consistently obtain PFC responses, a 4 wk period must elapse between priming and boosting, while boosting is generally ineffective after the 9th wk. Some of these features are explicable in terms of a very small precursor pool for the ABA epitope. Priming expands the pool of responsive cells, an interpretation which is consistent with the appearance of ABA-specific RFC after priming, and the expanded memory cell population produces IgG antibody after secondary stimulation. The regulatory mechanism that limits the functional lifetime of ABA-specific memory cells to approximately 1–2 mo is unknown.

It is noteworthy that an anti-ABA IgM response to ABA-Ficoll has been obtained in CBA/N mice using a tripeptide spacer between the ABA group and the carrier (24). Spacers of this type have been shown to influence the class of antibody formed against the phosphorylcholine epitope (25). Therefore, the possibility remains that the IgM antibody in that study was directed against at least a portion of the spacer peptide as well as the ABA group. It would be of considerable interest to know if these anti-ABA PFC reside in the CRL(-) B-cell compartment.

An additional noteworthy finding in the present investigation is that the 7S as well as the 19S PFC response to DNP-Ficoll was unaffected by C3 depletion, in contrast to the 7S PFC response to DNP-BGG. This observation, together with the almost exclusive residence of the in vitro anti-DNP-Ficoll response in the CRL(-) B-cell fraction, indicates the two IgG responses have different signalling or triggering requirements, one in which C3 is obligatory and the other in which it is apparently uninvolved. We interpret the findings to mean that clearly distinctive B-cell subpopulations respond to T-dependent and to true (nonpolyclonal mitogen) T-independent antigens.

The response of CRL(-) B cells to SRBC further complicates the picture. Since this response was sensitive to anti-Thy-1 serum and contamination of the CRL(-) cultures by CRL(+) B cells could not have accounted for its magnitude, it seems clear that at least a subpopulation of CRL(-) B cells is capable of cooperating with T cells in response to T-dependent antigens. This conclusion is consistent with observations of Pepys (7), who found significant residual antibody levels in animals depleted of C3 by CoV before immunization with SRBC, and of Parish (26) and Parish and Chilcott (27), who reported that RBC were

capable of stimulating both CRL(+) and CRL(-) B cells in the presence of a T-cell signal. Of interest, in the adoptive secondary response to horse RBC, at least 50% of the responsive B cells were in the CRL(-) population (26), whereas in the present investigation the CRL(+) population housed approximately six times as many precursors for SRBC as did the CRL(-) population. This difference may be time related, since Parish and Chilcott allowed at least 3 wk between priming and boosting while we allowed only 4 days. It is conceivable that the rate of generation of B-cell memory differs in the two populations.

Models for B-cell activation have generally postulated a single class of B cells capable of responding to both T-dependent and T-independent forms of antigen (1). Thymus dependency requires a minimum of two signals, one provided by the antigen and a second derived from the T cell. According to this model, T-independent antigens functionally bypass T cells by generating both signals. While this may be true, for example, in the triggering of CRL(+) B cells by polyclonal mitogens such as LPS and polymerized flagellin (28), it is a less attractive hypothesis for the activation of CRL(-) B cells by hapten-Ficoll conjugates. The advent of distinctive B-cell subpopulations with the properties described herein raises the possibility that they have entirely different signaling requirements. It is probably premature to speculate at this point about the number of signals generated by true T-independent antigens. However, a plausible working model based on the present findings and those reported elsewhere and discussed above is depicted in Fig. 6. CRL(+) B cells comprise about 80% of the splenic B-cell population, make only T-dependent responses, and are activated by dual signals provided by antigen and T cells, the latter via conversion of C3 to C3b. This signal is likely provided by a nonantigen-specific T-cell factor which can be replaced by any complement-activating molecule such as LPS. The CRL(-) B cells which make T-dependent responses are envisaged here as a subpopulation distinct from those which make true T-independent responses, although that has yet to be proven. A marker which can distinguish between the functional subsets of CRL(-) cells is required; a potential candidate may be the Ia antigen found on B cells (29). We propose that CRL(-), T-dependent B cells are activated by an antigen-specific T-cell factor via the Ia marker. This hypothesis is amenable to experimental test since anti-Ia antisera are available. The T-independent subpopulation of CRL(-) B cells have signaling requirements which can be satisfied by antigens of appropriate structure. LPS can activate CRL(+) and CRL(-) cells, but it is assumed that the effective signal it delivers to each type differs. Finally, it is unclear at present whether these B-cell subsets represent different terminal differentiation products or merely different stages in a single differentiation pathway, but this question, too, is amenable to experimental elucidation.

The almost total abrogation of T-dependent antibody responses by C3 depletion *in vivo* suggests that this complement component is an essential requirement for such responses. This raises interesting questions about the relationship between soluble helper factors released by activated T cells (30), C3, and B-cell activation. It has already been postulated that T cells may function in the cooperative process by releasing proteases which generate active split products of C3, and that the interaction of such products with B cells renders them

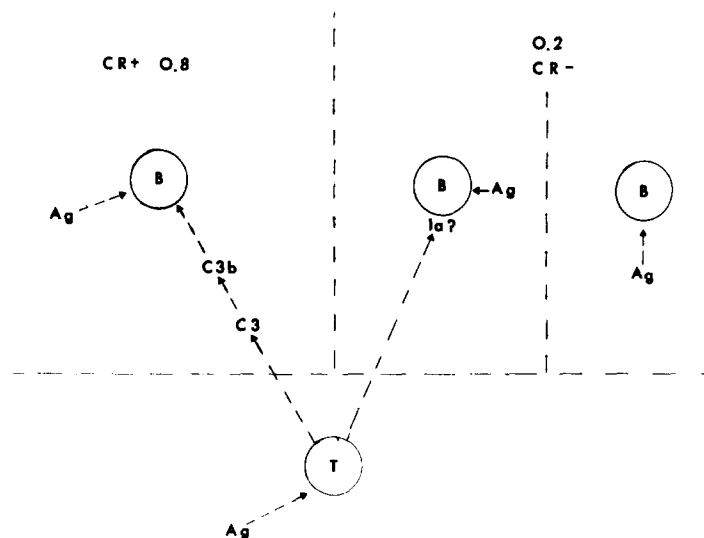


FIG. 6. A schematic model of putative B-cell subpopulations in the mouse and their signalling requirements.

susceptible to activation by T-dependent antigens (31). In that context, it would be of extreme interest to determine if purified helper factors from T cells degrade C3 and if split products of C3 can substitute for T cells in T-dependent antibody responses. Hartmann and Bokisch have recently shown that a relatively small fraction of murine B lymphocytes can be transformed by human C3b (32). More definitive information might be obtained in a homologous system in which specific antibody responses are assayed.

Summary

Strain A/J mice made secondary indirect plaque-forming cell (PFC) responses to azobenzene-arsenate (ABA) conjugates of giant keyhole limpet hemocyanin (KLH), a thymic-dependent antigen, but not to conjugates of Ficoll, a T-independent antigen. ABA-Ficoll was also unable to elicit a response in animals primed with ABA-KLH, which have an expanded anti-ABA memory cell pool. On the other hand, ABA-Ficoll rendered mice unresponsive to ABA-KLH when administered before priming or boosting with the T-dependent immunogen. Hence, the T-independent antigen was able to tolerize but unable to trigger B-memory cells responsive to the T-dependent antigen. A/J mice immunized with dinitrophenyl conjugates of Ficoll or bovine IgG (BGG) made vigorous IgM and IgG PFC responses. PFC responses to ABA-KLH and 2,4-dinitrophenyl (DNP)-BGG were abrogated by depleting mice of C3 with cobra venom factor, whereas the IgM and IgG PFC responses to DNP-Ficoll were unaffected. B lymphocytes were fractionated on the basis of receptors for C3 and the subpopulations were assayed for *in vitro* PFC responses to DNP-Ficoll. Very little response was obtained from complement receptor lymphocyte [CRL(+)] B cells, whereas CRL(-) cells were more responsive than unfractionated B cells. Both populations responded to a polyclonal B-cell mitogen (lipopolysaccharide). On the other

hand, the in vitro PFC response to a T-dependent antigen (sheep erythrocytes) correlated with the presence of CRL(+) B cells in the cultures. However, a minor component of this response, sensitive to anti-Thy-1 serum, was made by CRL(-) B cells, indicating the existence of subpopulations of T-dependent B cells with different signalling requirements.

The results suggest that most B cells responsive to T-dependent antigens possess receptors for C3 and that C3 plays an obligatory role in the response of these cells. A distinct subpopulation of B cells which lack C3 receptors respond to T-independent antigens. The precursors of PFC for the ABA epitope reside largely or exclusively in the CRL(+) compartment in A/J mice, whereas precursors for the DNP determinant are found in both compartments.

The authors gratefully acknowledge the assistance of Dr. Georges Der Balian and Miss Inge M. Stoltenberg.

Received for publication 9 April 1976.

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