# INFLUENCE OF THE MACROMOLECULAR FORM OF A B CELL EPITOPE ON THE EXPRESSION OF ANTIBODY VARIABLE AND CONSTANT REGION STRUCTURE

By SUZANNE FISH AND TIM MANSER

*From the Department of Molecular Biology, Princeton University, Princeton, New Jersey 08544*

Molecular analyses of murine humoral immune responses have been largely limited to responses induced by soluble proteins, polysaccharides, and hapten conjugates thereof (1–15). However, in nature the mouse rarely encounters an antigenic structure in association with such chemically simple forms, but rather in association with a viral particle, bacterium, or parasite. How such differences in the macromolecular form of an epitope affect the structural outcome of the humoral immune response to that epitope is not well understood.

Antigens may differ in their humoral immunogenic properties due to differences in epitope valence, solubility, mitogenic qualities, stability, degree of T cell independence, adjuvant properties, and ability to be phagocytized by macrophages. Any one of these factors could influence the structure of antibodies elicited to an epitope on the antigen due to resulting differences in the degree of participation in the immune response of T cells, accessory cells, or B cells that are members of functionally distinct subsets or reside in particular lymphoid microenvironments. Indeed, a number of investigations (2, 16–21) have revealed that the isotypic profile of serum antibodies elicited to haptens can vary dramatically depending on the molecular form of the haptenic determinant. Haptens conjugated to soluble proteins often elicit antibodies mainly of the IgG1 class, while the same haptens conjugated to polysaccharide often elicit IgM and IgG3 (2, 16, 17, 19). The immune response to viruses is nearly exclusively composed of IgG2a (22), and the immune response to many parasites is composed predominantly of IgE (23, 24). These data demonstrate that the various isotypic forms of Ig are not selected at random for expression but that the immune system is capable of distinguishing among a variety of macromolecular forms of an epitope and regulating expression of the antibody isotypes elicited to that epitope in response to such differences.

In contrast to the information available on the effects of antigenic form on the expression of Ig isotypes, little is known (2, 25, 26) about how this influences expression of particular antibody variable region structures. While the antigenic specificity of a V region clearly plays a role in the clonal selection process, it is not understood whether the V region structural outcome of an antigen-driven

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immune response can be modified by processes that act independently of the structure of the eliciting epitope. One of the factors that might well influence such processes is the macromolecular form of the antigen. To gain insight into this question we have conducted a molecular analysis of the humoral immune response of A/J mice to the hapten *p*-azophenylarsonate (Ars)[1] conjugated to the bacterial carrier *Brucella abortus* (Bruc) and compared the results with previous results (1) obtained from the analysis of the A/J immune response to this same hapten conjugated to a soluble protein carrier (Ars–keyhole limpet hemocyanin [KLH]).

## Materials and Methods

*Antigen Preparations.* Preparations of Bruc "tube antigen" (a heat-killed and phenol-preserved preparation of whole bacteria) were obtained from the New Jersey division of the U.S. Department of Agriculture. KLH was obtained from Calbiochem-Behring Corp., La Jolla, CA. Ars conjugates of both these antigens were prepared as previously described (27).

*Production of Hybridomas.* 8-wk-old female A/J mice (obtained originally from The Jackson Laboratory, Bar Harbor, ME) were injected intraperitoneally with 100 $\mu$l of a 1% suspension of Ars-Bruc in either CFA or PBS. Primary immune response hybridomas were produced from the spleen cells of mice taken 4 or 5 d after this single injection. Secondary immune response hybridomas were produced from the spleen cells of mice taken 3 d after a secondary 100-$\mu$l i.p. injection of a 1% suspension of Ars-Bruc in PBS administered 33–37 d after primary immunization as described above. Fusions were performed using the SP2/0 parental line as previously described (28).

*Screening of Hybridoma Populations.* Hybridomas expressing mRNA homologous to a $V_H Id^{CR}$-specific DNA probe were identified using the lysate hybridization technique (28). mAbs produced by $V_H Id^{CR}$-expressing hybridomas were assayed for Ars-binding activity and expression of various idiotypic determinants as described previously (29).

*RNA Purification, Sequencing, and Blot Analysis.* Total hybridoma RNA was purified using the guanidine isothiocyanate lysis/CsCl gradient procedure (30, 31). Poly(A)$^+$ RNA was purified using oligo(dT) cellulose chromatography. The oligonucleotide-primed dideoxynucleotide mRNA sequencing technique was used to determine the sequences of expressed $V_H$ and $V_L$ genes (32, 33). Oligonucleotide primers specific for the $C_\mu$, $C_{\gamma 3}$, $C_{\gamma 1}$-$C_{\gamma 2a}$-$C_{\gamma 2b}$, and $C_\kappa$ genes were used, as well as a primer that is specific for an internal site in the $V_H Id^{CR}$ gene segment. Northern blot analysis of total hybridoma RNA was performed as described (34).

*mAb Purification, Isotyping, and Ars Affinity Measurement.* Isotypes of mAbs were determined using hybridoma culture supernatants and an ELISA mouse antibody subtyping kit (Boehringer Mannheim Diagnostics, Inc., Houston, TX). mAbs were purified from culture supernatants using Ars-bovine gamma globulin/Sepharose affinity chromatography. After supernatants were loaded, the columns were washed extensively, first with 0.05% Tween-20 in PBS and then with 3 M urea in 10 mM Tris (pH 8.0). Antibody was eluted from the column using 2 M KSCN and the eluate was immediately dialyzed against PBS. Ars affinities of mAbs were determined via fluorescence quenching essentially as described previously (35). Measurements were done at 22°C using a fluorescence spectrophotometer (model MPF-66; Perkin-Elmer Corp., Norwalk, CT). The free ligand concentration at 50% of the maximum quench was used to determine association constants.

*Serology.* Primary Ars-Bruc immune sera were obtained on days 7, 17, and 28 after intraperitoneal injection of five female A/J mice with 100 $\mu$l of a 1% suspension of Ars-Bruc in PBS. Secondary Ars-Bruc immune sera were obtained from five female A/J mice 10 d after a secondary 100-$\mu$l i.p. injection of 1% Ars-Brucella (after an identical primary

[1] *Abbreviations used in this paper:* Ars, *p*-azophenylarsonate; Bruc, *Brucella abortus* "tube" antigen; KLH, keyhole limpet hemocyanin; TD, T cell dependent; TI, T cell independent.

immunization and a 38-d rest period). Secondary Ars-KLH immune sera were obtained from 16 female A/J mice 10 d after a 100-$\mu$g i.p. injection of Ars-KLH in PBS (after a primary 150-$\mu$g i.p. injection of Ars-KLH emulsified in CFA and a 30-d rest period). Quantitation of Ars-binding antibodies and idiotype in immune sera was performed as previously described (29). Quantitative determination of the isotype distribution of Ars-binding antibodies in immune sera was determined as follows. Serum samples were pooled and dilutions made in 10 mg/ml BSA/0.05% Tween-20 in PBS. Ars-binding antibodies in serum dilutions were then affinity purified by incubation on Ars-BSA-coated 96-well polyvinyl chloride plates. After washing, appropriate dilutions (in the buffer described above) of rabbit antisera specific for mouse IgM, IgG3, IgG1, IgG2a, or IgG2b (Litton Bionetics, Kensington, MD) were incubated in the wells. After washing, rabbit antibody bound to the wells was elaborated using a $^{125}$I-labeled goat anti–rabbit Ig reagent (Southern Biotechnology Associates, Inc., Birmingham, AL) and the wells were cut and counted using a gamma counter (model NE1600; Nuclear Enterprises America, Fairfield, NJ). Isotype content was calculated by comparing the isotype titration curves generated from serum samples with those generated using previously characterized Ars-binding mAbs (all having similar affinities for Ars) of the appropriate isotypic class.

## Results

We have previously reported (1) the results of a molecular analysis of the humoral immune response of A/J mice to Ars-KLH. A major fraction of the anti-Ars mAbs expressed by hybridomas isolated early (days 3–9) in the anti-Ars-KLH response are encoded by a single $V_H$ gene segment ($V_HId^{CR}$) in combination with a diverse assortment of D, $J_H$, $V_L$, and $J_L$ gene segments. These antibodies have low affinities for Ars on average ($K_a$ from $<5 \times 10^4$ to $5 \times 10^5$/M) and their V regions have not been structurally altered by somatic mutation. $V_HId^{CR}$-expressing hybridomas isolated at later times (days 10–21) during the primary Ars-KLH response (Manser, T., unpublished results) and during the secondary response (1) are characterized by restricted gene segment combinational diversity (a V domain encoded by a single combination of gene segments termed the "canonical" combination predominates), extensive somatic mutational alteration, and increased (as compared with early primary antibodies) affinity for Ars ($K_a$ from $10^5$ to $10^7$/M). We previously suggested that an antigen-driven process of V gene somatic mutation and clonal selection of B cells expressing high-affinity surface Ig can best explain this maturation of the anti-Ars-KLH response (36).

We wished to determine whether altering the macromolecular form of Ars would result in alteration of the structural maturation pathway of $V_HId^{CR}$-encoded antibodies, described above, that had been seen to occur during the Ars-KLH response. We chose to use Bruc as an alternate Ars carrier in these experiments for two reasons. First, Bruc is a particulate, complex bacterial antigen that has been categorized as a T cell–independent (TI) type I antigen (37) and hence is very different in its immunogenic properties from KLH (a T cell–dependent (TD) soluble protein antigen). Second, serological analyses of the strain A immune response to Ars-Bruc have been previously reported by Lucas and Henry (38) and Conger et al. (39). Both groups found that antibodies bearing idiotypic determinants characteristic of antibodies encoded by the $V_HId^{CR}$ gene segment ($Id^{CR}$ determinants) were elicited to Ars conjugates of Bruc.

Hybridomas were produced from the splenic B cells of five A/J mice on days 4 and 5 of the primary Ars-Bruc response and from six mice on day 3 of the secondary response. Since our analysis of the Ars-KLH response used mice that

```
                         20                                           -30-----CDR1-------                     40
MH Vh      --- --- --- --- C-- --- --- --- A-- --- --- --- --- --- --- --- --T --- --- --- --- --- --- --- --- ---
VhIdCR     TCC TCA GTG AAG ATG TCC TGC AAG GCT TCT GGA TAT ACA TTC ACA AGC TAC GGT ATA AAC TGG GTG AAA CAG AGG CCT
 1ABA2'-1  --- --- ---  -- --- --- -        -- --- --- --- --- --- -T- --- --- --- --- --- --- --- --- --- --- ---
 2ABA2'-2  --- --- --- --- --- --- --- --- --- ---  -- --- -A- A-- --- --- --- --- --- --- --- --- --- --- --- ---
 3ABA2'-4      --- --- --- --- --- --- ---  -- --- --- --- -T- --- --- G-- --T --- --- --- --- ---  -- --- --- ---
 4ABA2'-6  --- --- --- --- --- --- - - -- A-- --- - - --- --- --- --- --T --- - - --- --- --  --- --- --- --- ---
 5ABA2'-7      --- --- --- --- --- --      -- - - --- --- --- --- --- --- --- --- --- --- --- --- ---  -- --- --- ---
 62C2A         --- ---  -- --- --- --- --       - --- --- --- --- --- --- ---  -- --- --- --- --- ---  -- - -  -- ---
 72P2C         ---   -  -- --  --- -      -     - --- - - --- --- --- --- ---  -- - - --- --- -   ---  -- --- --  ---
 82P2A     --- --- ---  -- --- --- --- --  -- --- --- --- T-- --- --- --T A-- --- --- --- --- --- --- --- --- ---
 9ABA2'-3                  --- --- --- --- --- --C --C --T --- --C --- --- T-- --- --- --- --- --G --- --  ---
102P1A1    --- --- --- --- --- --- --- -C- A-- --- - - --- --- --- --- --- --- --- --- --- --- --- --- --   --
112P1C                 --- --- - - --- - - --- - - --- --- --- --- --- - - --- --T -   ---  -- --- --   -
122C3A         --- --  --- --- --- - - --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
--------------------------------------------------------------------------------------------------------------------
131P1C     --- --- --- --- --- --- -    --   - --- - - --- --- --- --- --- --- --- --- --- --- ---  -- --- --- --
141P2A      -- --- --- --- --- --- -    -- - - --- --- --- --- --- --- --- --- --- --- --- ---  -- --- --- ---
151P2E         --- ---  --  -- --- - - --   - --- --- --- --- --- --  --- --- --- --- --- --- ---  -- --- ---  -
161C4E      -- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
                                                                                 17ABA5-1 --- --- ---  --
18ABA8-1       --- ---  -- --- --- ---  -  - - --- - - --- --- --- --- --- --- - - --- --- --  ---  -- --- --  --


                                                        T to I
                                                          58
                                      -50----------------------------CDR2-----60--------------------               70
  --- --- --- --- --- --- --- --- --- --- T-- --- --- --- --- --- --- GC- --- --- --- C-- --- --- --- --- G-- --- ---
  GGA CAG GGC CTG GAA TGG ATT GGA TAT ATT AAT CCT GGA AAT GGT TAT ACT AAG TAC AAT GAG AAG TTC AAG GGC AAG ACC ACA CTG
1 --- --- --- --- --- --   -- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --  --- --- --- ---
2 --- --- --  --- --- --- --- --- --C --A --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
3 --- --- --  --- --- --- --- --- --- --- --- --- --  --A --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
4 - - ---     --- --- --  --- --- --- --- --- --- --- --- --- --- -T- --- --T --- --- --- --- --- --- --- --- --- ---
5 --- --- --  --- --- --- --- --- --- --- --- --- --- --- --- --- -T- --- --- --- --- --- --- --- --- --- --- --- ---
6 --  - - -   --- - - --   -- --- --- --- --- --- --- --- --- --- -T- --- --T --- --- --- --- --- --- --- --- --  ---
7     ---      -  -- --  --- - - --- --- --- --- -   ---  -- --- -T- --- ---  -- --- --- --- --- --  --- --- --- ---
8 --- ---     --- --- --  --- --- --- --- --- --- --- --- --- --- -T- --- --- --- --- --- --- --- --- --- --- --G ---
9 --- --A --- --T --G --- --- --- A-- --- T-- --- --T -G- A-- AG- --- --C --- --- --- --- --- --- A-- --- G-- --- ---
10- - ---     --- --- --  --- --- --- --- --- --- --- --- -T- --- --- --- --- --- --- --- --- --- --- --- T-- --- ---
11    ---     ---  - --   -- - - --- --- --- --- --- C-- --- --- -T- --- --- --- --- --- --- --- --- --- --- --G ---
12--- ---     --- --- --  --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --  --- --- --- ---
--------------------------------------------------------------------------------------------------------------------
13--  ---     --- --- --  --- --- --- --- --- --- --  --- --- --- --- --- --- --- --- --- --- --- --  --- --- --- ---
14-   ---     --- - - --  --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --   -- --- --- ---
15 -   - -    - - - - --  --- --- --- --- --- --- --- --- --- --- ---  -- --- --- --- --- --- --- ---  --  -- --- ---
16--- ---     --- --- --   -- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
17--- ---     --- --- --   -- - - --- --- --- --- -   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
18- - ---     ---  -- --   -- - - --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
                                                                    191P1A   --- --- --  -    -- --- --- --
          201C4A--   -- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --  --  --- ---
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FIGURE 1. Nucleotide sequences of the $V_HId^{CR}$ gene segments expressed by Ars-Bruc primary and secondary hybridomas.[2] The sequences are compared with the sequence of the germline $V_HId^{CR}$ gene segment (*VhIdCR*) and another gene segment (*MH Vh*) thought to be the most homologous $V_H$ gene to $V_HId^{CR}$ in the A/J genome. Sequence identity is indicated by a dash. Nucleotide differences are shown explicitly. Nucleotide positions that could not be unequivocally identified are indicated by a gap. The position of complementarity determining region (*CDR*) coding sequences are shown above the sequences as are the positions of amino acid codons, numbered sequentially from the mature $NH_2$-terminus, of the encoded polypeptide chain. The sequences are divided into two groups: those obtained from secondary hybridomas, shown immediately below the germline $V_HId^{CR}$ sequence, and those obtained from primary hybridomas, shown below and separated by a solid line from the secondary sequences. The names of the hybridomas that express each of the $V_H$ genes are indicated at the 5′ end of each sequence and these names are preceded by a number (1–12 for secondary hybridomas and 13–18 for primary hybridomas) that also precedes the continuation of the sequences for ease of alignment. Amino acid codon 58, which is often observed to have mutated from a threonine to an isoleucine codon, is shown. Figure continued on facing page.

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[2] These sequence data have been submitted to the EMBL/GenBank Data Libraries under the accession number Y00633.

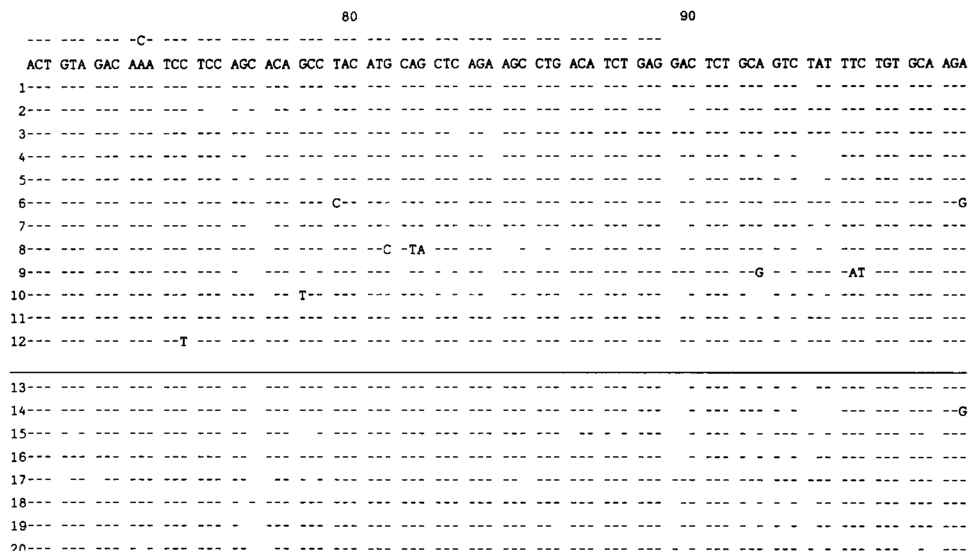

FIGURE 1 (*continued*)

had been primed with Ars-KLH in CFA, a group of mice were primed with Ars-Bruc in CFA to control for possible adjuvant effects. The resulting hybridomas were screened for expression of mRNA homologous to the $V_HId^{CR}$ gene segment (see Materials and Methods). Hybridomas identified in this way were then subcloned and the sequences of their expressed V region genes were determined. All of the hybridomas that were further characterized produce Ars-binding antibodies.

Figs. 1 and 2 present the sequences of the $V_H$ genes expressed by these hybridomas as compared with the sequence of the $V_H$ gene expressed by 36-65, a hybridoma isolated during the A/J anti-Ars-KLH response that expresses the canonical combination of V gene segments ($V_HId^{CR}$, $D^{CR}$, $J_H2$, $V_\kappa Id^{CR}$, and $J_\kappa 1$) in unmutated form. The primary hybridomas express the $V_HId^{CR}$ segment in association with a diverse collection of D and $J_H$ segments. Most of these gene segment combinations are similar to those previously observed (1, 13) to be expressed by primary hybridomas isolated from the Ars-KLH response. All of the primary Ars-Bruc hybridomas express the $V_HId^{CR}$ gene segment in unmutated form. A single primary hybridoma (1P1C) was found to express the canonical $V_H$-D-$J_H$ combination. Less extensive analysis of the $V_L$ genes expressed by the primary hybridomas revealed that several express $V_L$ genes related or identical to the $V_\kappa Id^{CR}$-$J_\kappa 1$ combination (including 1P1C), but many express unrelated $V_L$ genes (see Table I). The observation that hybridomas expressing the canonical combination of V gene segments can be isolated from both the anti-Ars-KLH and anti-Ars-Bruc response strongly suggests that the antigenic structure of Ars in the context of these two carriers is similar if not identical (i.e., carrier components do not contribute differentially to the Ars antigenic determinant).

The extensive combinational diversity expressed in the context of the $V_HId^{CR}$ gene segment among primary hybridomas was not as apparent among the secondary Ars-Bruc hybridomas (Fig. 2 and Table I). Three examples of hybri-

```
      VH                         D                               JH
                       100                             107
36-65  GCA AGA     TCG GTC TAC TAT GGT GGT AGT TAC     TAC TTT GAC TAC TGG GGC CAA GGC ACC ACT CTC ACA GTC TCC TCA JH2
ABA2'-4--- ---     --C TAT --- --- --- --- --C ---     GTA --- --- --T --- --- --- --- --- --- --- --- --- ---     JH2
ABA2'-6--- ---     --C CAT --- --- --- --- --C -X-     -C- --- --- --- --- --- --- --- --- --- --- --- --- --- --- JH2
ABA2'-7--- ---     --C CAT --- --- --- --- --C ---     -C- --- -X- --- --- --- X-- --- --- X-- --- --- --- --- --- JH2
2P1A1  --- ---     --- AAA --T --- --- --- C-A CG-         X-- --- --- --- -X- XX- --- ---                         JH2
2P1C   --- ---     --- AAT --- --- --- --A C-C CG-         --- --- --- --- --X A-X --- --- --- --- ---             JH2
2C2A   --- --G     AGC     --- --- --- --C TCX GG-  ^  --- --C --T GT- --- --- AC- --G ---                         JH1
                                                /TAC TGG\
2P2C   --- ---     AGT     --- --- --- --- --C -T- TGG --- --C --T GTG --- --T ACX --T                             JH1
ABA2'-3--- ---             --- --- --- --- --G --T  ^  GCT A-G --- --- --- --T --- --A --- T-A G-- --C --- ---     JH4
                                                /TAC TAC\
ABA2'-1--- ---     CGA                                 --- --C --T GT- --- --- AC- --G --- --G G-- --X --- --- --- JH1
2P2A   --- ---     AT-                                 XGG --- XCT X-- X-- --- -X- --                              JH3
ABA2'-2--- ---     ATA TAT --T                             --- -CT --- --- --- --- --G --T CTG G-- --T             JH3
2C3A   --- ---     AGA -GA GGA G-- -A- --X TC- CGX  ^  GCT A-G --- --- --- --T --- --X --- T-A                     JH4
                                                /TAC TAT\
---------------------------------------------------------------------------------------------------------------------
1P1C   --- ---     --T AGG --- --- --- --- --C ---     --- --- --- --- --- ---                                     JH2
1C4E   --- ---     --A TAT --- --- --- --- --C --T     GCT A-G --- --- --- --T -X- --X X-- T-A GX-                 JH4
ABA8-1 --- ---     ATC                                 -GG X-- -CT --- --- ---                                     JH3
1P2A   --- --G     G-C                                 -XG --- -CT --- --- --- -X- --G --T CTG                     JH3
1P2E   --  ---     -GT -AT XTT ACT -GA                 GCT A-G -X- --- --- --T -X- --X                             JH4
ABA5-1 --- ---     --T TA- C-T --T A-- AAC TAC GG- TAT GCT A-G --- --- ---                                         JH4
1P1A   --- ---     GXX GXX ATT --- -A- --- TAC ---  ^  -XX X-- -XT --- --- --- ---                                 JH3
                                                /GGG GXC\
1C4A    -  ---     --- AGG XGT -X- -XA -XC TXC             --- -X-                                                 JH2
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FIGURE 2. Nucleotide sequences in the $V_H$-D-$J_H$ region of the $V_H$ genes expressed by secondary and primary Ars-Bruc hybridomas. The sequences are compared with the sequence in this region of the $V_H$ gene expressed by the hybridoma 36-65, a hybridoma that expresses the canonical combination of V gene segments in unmutated form. Sequences are aligned to maximize homology to the 36-65 sequence. Sequence identity is indicated by a dash, differences are shown explicitly. Nucleotide positions that could not be unequivocally determined are indicated by an **X**. Sequences obtained from secondary Ars-Bruc hybridomas are shown immediately below the 36-65 sequence. Sequences derived from primary Ars-Bruc hybridomas are shown below the secondary sequences and are separated from these sequences by a solid line. Two amino acid codons that are observed to vary among the $V_H$ genes expressed by Ars-KLH hybridomas that express the canonical combination of gene segments (amino acid positions 100 and 107) are indicated.[3]

domas expressing the canonical combination of gene segments were found, and more restricted use of D, $J_H$, and $V_L$ segments than observed among primary hybridomas is apparent. In addition, all of the secondary hybridomas express somatically mutated forms of the $V_H Id^{CR}$ gene segment (Fig. 1). Two of the hallmarks of antigenic selection of V region somatic mutants are evident in these gene segments; productive mutations are clustered in and around complementarity determining regions, and the same productive mutations are found at identical positions in genes expressed by independently isolated hybridomas (e.g., $V_H$ position 58). Sequences of several of the $V_L$ genes expressed by $V_\kappa Id^{CR}$-expressing secondary hybridomas were determined and are consistent with somatically mutated forms of the $V_\kappa Id^{CR}$ gene segment (reference 40; Wysocki, L., personal communication) (see Table I).

These data suggest that an antigen-driven process of somatic mutation and clonal selection of B cells expressing high-affinity surface Ig entirely analogous

[3] These sequence data have been submitted to the EMBL/GenBank Data Libraries under accession number Y00634.

TABLE I
*Characteristics of mAbs Produced by Primary and Secondary Ars-Bruc Hybridomas*

| Exp. | Hybridoma | Mouse number | $V_H Id^{CR}$ mutation | $V_L$ homology $V_\kappa Id^{CR}$ | Isotype | Affinity for Ars |
|---|---|---|---|---|---|---|
| | | | | | | $M^{-1}$ |
| 1 | ABA5-1 | 1 | No | No | IgG2b | $2.4 \times 10^5$ |
| | ABA8-1 | 2 | No | No | IgG2a | $<8 \times 10^4$ |
| | 1P1A | 3 | No | No | IgM | |
| | 1P1C | 3 | No | Yes | IgG3 | $3.8 \times 10^5$‡ |
| | 1P2A | 4 | No | No* | IgM | $<7 \times 10^4$ |
| | 1P2E | 4 | No | No | IgG2a | $<6 \times 10^4$ |
| | 1C4A | 5 | No | No | IgM | |
| | 1C4E | 5 | No | Yes | IgG2a | |
| 2 | 2P1A1 | 6 | Yes | Yes§ | IgG2a | |
| | 2P1C | 6 | Yes | Yes | IgG2a | |
| | 2P2A | 7 | Yes | No | IgG2a | $1.4 \times 10^5$ |
| | 2P2C | 7 | Yes | Yes§ | IgG3 | $5.9 \times 10^5$ |
| | ABA2'-1 | 8 | Yes | No | IgM | |
| | ABA2'-2 | 8 | Yes | No | IgG2a | |
| | ABA2'-3 | 9 | Yes | No | IgG2b | |
| | ABA2'-4 | 9 | Yes | Yes | IgG2b | $1.0 \times 10^7$‡ |
| | ABA2'-6 | 9 | Yes | Yes | IgG2a | $\sim 1 \times 10^7$‡ |
| | ABA2'-7 | 9 | Yes | Yes | IgG2b | $8.8 \times 10^6$‡ |
| | 2C2A | 10 | Yes | Yes§ | IgG2a | $5.9 \times 10^6$ |
| | 2C3A | 11 | Yes | No | IgM | $<2 \times 10^4$ |

Mice 1–4 and 6–9 received a primary injection with Ars-Bruc in PBS. Mice 5, 10, and 11 received Ars-Bruc in CFA. Data obtained from primary anti-Ars-Bruc antibodies are shown in Exp. 1, those obtained from secondary anti-Ars-Bruc antibodies are shown in Exp. 2. The presence or absence of somatic mutations in the $V_H Id^{CR}$ gene segment encoding these antibodies was determined as described in Materials and Methods and in the Legend to Fig. 1. Homology of the $V_L$ genes encoding these antibodies was determined either by Northern blot analysis using a $V_\kappa Id^{CR}$ probe or by nucleotide sequencing of the $V_\kappa$ genes encoding these proteins.

* This sequenced $V_\kappa$ was not homologous to the $V_\kappa Id^{CR}$ gene segment but was highly homologous to the $V_\kappa 36$–$60$ (13) gene segment.

‡ Affinities of some of the monoclonal antibodies for Ars-tyrosine were determined as described in Materials and Methods. The footnote indicates affinities determined for antibodies that are encoded by the canonical combination of V region gene segments.

§ Sequenced $V_\kappa$ genes that appeared to be somatically mutated forms of the $V_\kappa Id^{CR}$ gene segment in combination with $J_\kappa 1$.

to the process thought to be responsible for the maturation of the Ars-KLH response also occurs during the Ars-Bruc response. A prediction of this hypothesis is that secondary Ars-Bruc hybridomas should produce mAbs that, in general, have higher affinity for Ars than do antibodies expressed by primary Ars-Bruc hybridomas. The Ars affinities of several of the antibodies produced by primary and secondary Ars-Bruc hybridomas were determined and this was found to be the case (see Table I). In particular, the secondary hybridomas that express the canonical combination in somatically mutated forms (ABA2'-4, ABA2'-6, and ABA2'-7) produce antibodies that have Ars affinities 2–10-fold higher than the antibody produced by the single primary hybridoma (1P1C) that expresses this combination of segments in germline form.

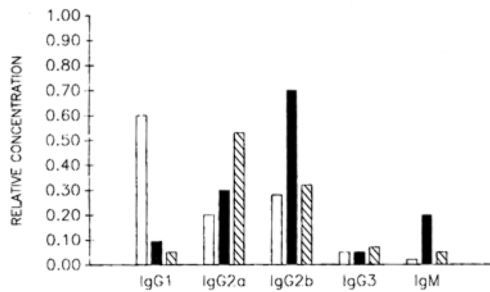


FIGURE 3. Isotype distribution of secondary anti-Ars serum antibodies. Mice were immunized, serum samples were taken, and isotype distribution of anti-Ars antibodies were determined as described in Materials and Methods. The results are presented as percentage of total serum anti-Ars antibody accounted for by either IgM, IgG3, IgG1, IgG2a, or IgG2b. The values for individual sera add up to slightly >100% due to mild crossreactivities of the antiisotype reagents. Sera were obtained from mice after either priming with Ars-KLH in CFA and boosting with Ars-KLH in PBS (□), priming with Ars-Bruc in PBS and boosting with Ars-Bruc in PBS (▨), or priming with Ars-Bruc in CFA and boosting with Ars-Bruc in PBS (■).

In contrast to the overall similarities of V region structure and maturation observed between the primary and secondary groups of $V_H Id^{CR}$-encoded antibodies elicited with Ars-KLH and Ars-Bruc, the predominant isotypic classes of anti-Ars mAbs elicited by secondary immunization with these two antigens differ. The majority of Ars-KLH-induced secondary hybridomas produce antibodies of the IgG1 class (1, 41), whereas most Ars-Bruc-induced secondary hybridomas produce either IgG2a or IgG2b (see Table I).

We wished to determine whether the predominant expression of IgG1 among Ars-KLH-induced secondary hybridomas, and IgG2 among Ars-Bruc-induced secondary hybridomas was reflective of the isotypic profile of secondary anti-Ars serum antibodies. Three groups of A/J mice were primed with either Ars-KLH in CFA, Ars-Bruc in CFA, or Ars-Bruc in PBS. 1 mo later the Ars-KLH mice were boosted with Ars-KLH in PBS and both groups of Ars-Bruc mice were boosted with Ars-Bruc in PBS. 10 d later all groups of mice were bled and serum samples from each group were pooled. The percentage of IgM and the various IgG isotypes among the anti-Ars antibodies in each pooled serum sample was then determined as described in Materials and Methods. The results presented in Fig. 3 show that IgG1 is the predominant isotype among secondary anti-Ars antibodies induced with Ars-KLH, whereas IgG2a and IgG2b predominate among secondary antibodies induced with Ars-Bruc. Fig. 3 also shows that the relative proportion of IgG2a and IgG2b found among Ars-Bruc-induced anti-Ars serum antibodies is influenced by whether the primary immunization was of Ars-Bruc emulsified in CFA or Ars-Bruc in PBS.

## Discussion

Our results suggest that differences in the macromolecular form of Ars have much more dramatic effects on the isotypic profile of the anti-Ars response of A/J mice than they do on the repertoire of V domains elicited to this haptenic determinant. In addition, the maturation of anti-Ars V region structure previously observed to occur during the course of the anti-Ars-KLH response also occurs in response to Ars-Bruc. The data suggest that regulation of isotype expression during a humoral immune response need not have an effect on V domain expression, diversification, and selection. More generally, isotype regu-

lation may be particularly sensitive to the macromolecular form of an epitope, while V region regulation is particularly sensitive to its molecular structure.

Our finding that secondary anti-Ars-Bruc antibodies comprise mainly IgG2a and IgG2b is reminiscent of the results of a previous study on the immune response to trinitophenyl-Bruc (42). However, such an isotypic profile, and our finding of somatic mutational diversification of V region structure during the Ars-Bruc response, are not consistent with many previous analyses of TI immune responses (2, 16–19). In fact, it has been argued that Bruc, while able to elicit an immune response in nude mice, nevertheless bears one of the hallmarks of a TD antigen; it can induce a state of immunological memory in normal mice (reference 43; Fish, S., and T. Manser, unpublished results). These considerations point out that in the presence of a functional T cell compartment it may be an oversimplification to consider any antigen truly T cell independent (44, 45).

Although the expression and diversification of Ars-specific V region structure observed to occur during the Ars-KLH and Ars-Bruc responses are very similar, several subtle differences are noteworthy. First, the absolute amount of somatic mutational alteration of the V region genes expressed by secondary Ars-Bruc hybridomas is somewhat less than that previously observed (1) among the V genes expressed by secondary Ars-KLH hybridomas (1.5 vs. 3% $V_HId^{CR}$ nucleotides mutated). In addition, the degree of predominance of expression of the canonical combination of gene segments among these two groups of hybridomas differs. 3 of 12 Ars-Bruc secondary hybridomas express this combination, whereas our previous analysis (1) of Ars-KLH secondary hybridomas revealed that 7 of 9 expressed this combination of gene segments. These data suggest that the maturation of anti-Ars V region structure may not progress to as great a degree in response to Ars-Bruc. This may be due, in part, to the fact that the anti-Ars response elicited by administration of Ars-Bruc in PBS (from which the major fraction of our hybridomas was derived) results in an anti-Ars response that is approximately fivefold lower in magnitude (as measured by levels of serum antibody) than the anti-Ars response elicited by Ars-KLH in CFA (data not shown). These quantitative differences apparently cannot account for the differences in the isotypic profiles of the two anti-Ars responses, since when Ars-Bruc is administered in CFA an anti-Ars serum antibody response results that is similar in magnitude to that obtained with Ars-KLH in CFA (data not shown). The isotypic profile of this Ars-Bruc in CFA–primed secondary Ars-Bruc response is characterized by the predominance of IgG2a and IgG2b (Fig. 3). In addition, despite the differences in magnitude of the responses, similar increases in Ars affinity between antibodies produced by primary and secondary hybridomas isolated from the Ars-Bruc and Ars-KLH responses are observed.

Ars-KLH–induced anti-Ars antibodies encoded by $V_HId^{CR}$ are characterized by the presence of a $V_H$-D junctional serine residue (46). Replacement of this junctional serine with alanine in a prototype canonical combination encoded V region via site-directed mutagenesis abolishes affinity for Ars (47). This result indicates that the reproducible occurrence of serine at the $V_H$-D junction of $V_HId^{CR}$-encoded Ars-binding antibodies results from antigenic selection. Strikingly, among 33 independently isolated anti-Ars-KLH antibodies examined to date all $V_H$-D junctional serines are encoded by the TCX group of serine codons;

the AGT or AGC serine codons are never observed. Many of the V regions expressed by the Ars-Bruc–induced $V_H Id^{CR}$-expressing hybridomas we have isolated also contain a TCX-encoded $V_H$-D junctional serine. We (46) and others (48) have argued that this biased codon usage may result from either nonrandom *de novo* junctional nucleotide addition or the occasional use of an additional TCX-containing D gene segment during the formation of $V_H$ genes that use the $V_H Id^{CR}$ gene segment. Apparently the TCX junctional codon is selected at both the protein and nucleic acid levels.

In contrast to the exclusive use of TCX to encode the $V_H Id^{CR}$-D junctional serine observed among previously characterized anti-Ars-KLH antibodies (46), two of the secondary anti-Ars-Bruc antibodies we have isolated have $V_H Id^{CR}$-D junctional serine residues encoded by either AGT (2P2C) or AGC (2C2A). Both these hybridomas express V gene segment combinations that are close approximations to the canonical combination (i.e., they express $V_H Id^{CR}$, $D^{CR}$, $V_\kappa Id^{CR}$, and $J_\kappa 1$ but differ in expressing $J_H 1$). Interestingly, in the D regions of the $V_H$ genes these two hybridomas express, position 100 (the first codon 3′ of the junctional serine codon) appears to be derived from the germline $D^{CR}$ gene segment (49). In the D regions of the $V_H$ genes expressed by Ars-KLH hybridomas, the TCX serine codon is nearly always followed by a codon of variable sequence, suggestive of random nucleotide addition 5′ to $D^{CR}$ coding sequence. Thus, the position 99 AGT and AGC codons in the $V_H$ genes expressed by the 2C2A and 2P2C hybridomas appear to have resulted from random nucleotide addition at the junction of the $V_H Id^{CR}$ and $D^{CR}$ segments during gene segment joining. Why hybridomas producing Ars-binding antibodies encoded in this way have never been isolated from the anti–Ars-KLH response is unclear. One possibility is that Ars-Bruc can stimulate a B cell subset that does not participate in the Ars-KLH response and differs from B cells that respond to Ars-KLH in expression of junctional diversity. Indeed, it has been suggested that different macromolecular forms of an epitope stimulate functionally distinct subpopulations of both naive (50) and memory (51) B cells. Further experiments are required to confirm these interesting but preliminary observations.

## Summary

We investigated the influence of the macromolecular form of an epitope on the structure of antibody variable and constant regions expressed by the B cell population participating in an immune response to that epitope. Hybridomas were constructed from strain A/J mice undergoing either primary or secondary immune responses to *p*-azophenylarsonate conjugated to Brucella abortus (Ars-Bruc). We determined the sequences of the V genes expressed by hybridomas selected on the basis of expression of a single $V_H$ gene segment known to encode a large family of anti-Ars antibodies. These sequences were compared with the sequences of V genes expressed by a previously characterized panel of hybridomas isolated in the same way during the primary and secondary responses of A/J mice to Ars-KLH. The repertoire of Ars-specific V domains expressed among primary and secondary hybridomas elicited with these two forms of Ars were similar, as were the differences between primary and secondary V region somatic mutational alteration and affinity for Ars. In contrast, predominant expression

of IgG2 anti-Ars antibodies was elicited in the secondary Ars-Bruc response, whereas secondary anti-Ars antibodies elicited with Ars-KLH are predominantly IgG1. Thus, differences in the macromolecular form of Ars clearly influence the isotypic profile of the anti-Ars response, but the expression, diversification, and selection of V domains elicited with this hapten are not greatly affected by such differences. Our results suggest that while isotype regulation is highly perceptive of the macromolecular form of a B cell epitope, V region regulation is primarily influenced by the molecular structure of that epitope.

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