INFLUENCE OF Igh-LINKED GENE PRODUCTS ON THE
GENERATION OF T HELPER CELLS IN THE
RESPONSE TO SHEEP ERYTHROCYTES*

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Since the cellular basis of antibody production was firmly established by Gowans
et al. (1), we have come to appreciate the intricacy of the cell interactions involved in
this process. Claman et al. (2), Davies et al. (3), and Miller and Mitchell (4) showed
that thymus-derived (T) cells had a synergistic (helper) effect on the generation of
antibody-secreting (B) cells. Mitchison (5) and Rajewsky and Rottlander (6) found
that help that could be generated via T cells responding to one set of antigenic
determinants while B cells were responding to others. These T cells will be designated
T-helper carrier or (THe).1

More recently, a second type of T-helper cell has been found. In certain mouse
strains, and under conditions in which production of an allotype had been suppressed,
Herzenberg et al. (7) showed that there are some T-helper cells (TH) that are
restricted to supplying help for the production of antibody of a particular isotype and
allotype. Subsequently, Hetzelberger and Eichman (8) and Woodland and Cantor
(9) were able to demonstrate the existence of TH with specificity for idiotype.
Kishimoto and Ishizaka (10) found that help for IgE could be generated separately
from help for IgG (10). Thus, TH with specificity for allotypes, idiotypes, and isotypes
have all been demonstrated. Collectively these will be called THIg.

What remains to be demonstrated is that THIg play an important role in the typical
situation where the initial response is clearly heterogeneous.

To examine this question we tried to determine if THIg are important in the
antibody response of CBA mice to sheep erythrocytes (SRBC). We also wanted to test
the idea that clones of THIg are selectively stimulated by an Ig-containing product of
an antigen-stimulated B cell. Finally, we wished to see if the production of antibody
of different isotypes was equally dependent on THIg.

We utilized a set of three congenic strains of mice that we have developed: CBA/
Tufts, CBA.nude, and CBA.Ighb. All three strains are H-2k and Mlsd. The first two
carry the IgH haplotype, whereas CBA.Ighb mice carry the IgH haplotype from

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Abbreviations used in this paper: PBS, phosphate-buffered saline; sIg, surface immunoglobulin; SRBC,
sheep erythrocytes; TH, helper T cells; THan, helper T cells specific for carrier determinants; THIg,
thymus-derived helper cells specific for immunoglobulin determinants.
C57BL/6 NIMR mice. The CBA.nude mice carry the nude gene and homozygous nu/nu mice lack a normal thymus.

In all of the experiments, the amount of help provided by T cells from Igh\textsuperscript{j} and Igh\textsuperscript{b} donors was compared. In essence, we asked if antigen-primed Igh\textsuperscript{j} B cells, but not Igh\textsuperscript{b} B cells, expand clones of THIg that help Igh\textsuperscript{j} B cells. The rationale for these experiments was that the Igh\textsuperscript{j} and Igh\textsuperscript{b} haplotypes are known to contain alternative alleles for the constant regions \(\mu, \delta, \gamma\), \(\gamma_2\), \(\gamma_2\), and \(\alpha\) (11). They also differ at some unknown number of V\(\beta\) loci (12). We reasoned that clones of THIg in mice of one strain should be selectively expanded by Ig-containing products produced by that strain. These expanded THIg clones should be able to help B cells that bear Ig molecules similar to those that expanded the clone. Therefore T cells primed by products of Igh\textsuperscript{j} B cells should be able to help B cells of Igh\textsuperscript{j} phenotype better than T cells primed by B cells of Igh\textsuperscript{b} haplotype.

Materials and Methods

**Animals.** CBA/Tufts, CBA.Igh\textsuperscript{b}, and CBA.nude mice were produced in our breeding colony at Tufts University School of Medicine, Boston, Mass. CBA/Tufts mice are derived from a cross between CBA/Ca NIMR and CBA/J mice. The congenic partner CBA.nude is in the 12th backcross generation. Nude mice and their phenotypically normal littermates were from the F\(_9\) to F\(_{12}\) generations. CBA.Igh\textsuperscript{b} mice are the product of 14 generations of backcrossing of the IgG\textsuperscript{b} allotype from C57BL/6 mice onto a CBA/Tufts background. Mice used in this study were from F\(_{14}\) to F\(_{18}\) generations. These mice are all H-2\(^a\) (J. Klein and H. H. Wortis, unpublished data) and Mls\(^a\) they do not provoke mixed lymphocyte responses (S. Tonkonogy, H. Winn, and H. H. Wortis, unpublished data). Skin grafts between CBA/Tufts and CBA.nude (+/?) mice are accepted in both directions. Grafts from CBA.Igh\textsuperscript{b} mice to CBA/Tufts or CBA.nude (+/?) mice are rejected slowly, indicating a weak histocompatibility barrier (H. Winn and H. H. Wortis, unpublished data). Grafts in the other direction are accepted. CBA.Igh\textsuperscript{b} mice bear the IgG\textsubscript{2b} and IgG\textsubscript{b} alleles. Their antibody to staphylococcal nuclease bears an Igh-Ns idiotype (13) produced by Igh-V\textsubscript{b} mice but not by mice with other Igh-V haplotypes (D. Sachs and H. H. Wortis, unpublished data). Because these mice bear the IgG\textsubscript{2b} allele and Igh-Ns allele and these two loci mark the known extremes of the Igh linkage group (13, 14), we concluded that these mice bear the whole Igh\textsuperscript{b} haplotype rather than assume that a double recombination event took place during the derivation of the congenic strains.

Unless otherwise indicated, female mice 2-5 mo of age were used. Nude mice were maintained in sealed, sterilized cages with filter tops. They received sterile bedding, food, and acidified water at 2-wk intervals. They were handled in a laminar flow hood under sterile conditions. Under these conditions, nude mice live for \(\geq 1\) yr.

**Antigens.** SRBC for immunization were purchased from Mr. Don Gaulitz of Massachusetts General Hospital, Boston, Mass. SRBC were kept in Alsever's solution at 4°C and used when 1-3 wk old.

**Immunization.** Experimental animals were challenged on days 0 and 21 with \(4 \times 10^7\) SRBC by intraperitoneal injection. As indicated, some T cell donor mice were immunized with \(4 \times 10^8\) SRBC by intraperitoneal injection 1 wk before they were killed. T cells from these mice are referred to as primed T cells.

**Cell Preparation and Transfer.** Lymphoid organs were pressed through a wire mesh and the cells collected were suspended in Hanks' balanced salt solution (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 2% fetal calf serum. Thymocytes were taken from 2-wk-old donors. Peripheral T cell-enriched preparations were made by removing surface Ig\(^{+}\) (slg\(^{+}\)) cells from lymph node suspensions by the panning technique (15, 16). Nonbinding cells were 2-5% slg\(^{-}\) as determined by fluorescein-coupled goat anti-mouse Fab prepared in this laboratory. They showed little mitogenic response to lipopolysaccharide (e.g., stimulation index = 6.5, \(\Delta\)cpm = 5,500 cpm) but did give a vigorous response to concanavalin A (e.g., stimulation
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index = 253, Δcpm = 251,000). 1 × 10^7 - 2 × 10^7 Ig^- (T) cells were injected i.v. into recipient nude mice as indicated. Recipient nude mice were tail bled on days 5 or 7 and day 12 or 13.

Antibody Assay. Serum antibodies to SRBC were measured in a radioimmunoassay developed by J. Haber and described in detail elsewhere (17). In brief 25 μl of serum diluted on 0.01 M phosphate-buffered saline (PBS) pH 7.5 was incubated with 25 μl of a 20% suspension of SRBC in round-bottomed microtiter plates (MIC-2000, Cooke Engineering Co., Alexandria, Va.) for 1 h at room temperature. The plates were spun at 180 g for 5 min. The supernates were removed, fresh PBS added, and the plates agitated. Washing was repeated three to four times.

II^-labeled goat anti mouse γ1, γ2a, γ2b, γ3, or μ antibody was added to the antibody-coated, washed cells. After an overnight incubation at room temperature, the plates were centrifuged and the antibody-coated cells washed three times. The wells were cut with a hot wire and the number of bound counts determined in a Beckman gamma counter (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.).

A serum pool from mice hyperimmunized with SRBC was used as a standard. Units of antibody in test bleeds were determined by comparison with the antibody content of the standard. Because no attempt was made to convert units into micrograms or moles of antibody, quantitative comparison of different isotypes can not be made.

Recent assays have utilized microtiter wells pretreated for 15 min at room temperature with 0.025 mg/ml poly-L-lysine hydrobromide (Miles Laboratories, Inc., Elkhart, Ind.). After coating, the wells were washed with PBS then incubated with 50 μl of 5% SRBC in 0.1 M PBS, pH 7.5, for 30 min. Excess cells were washed away. The assay then proceeded as above. This method of coating plates with SRBC has the advantage of permitting extensive washing without need for centrifugation, and gives results equivalent to those seen with the SRBC pellet method.

Goat Anti-Mouse Isotype. The preparation and characterization of the goat anti-mouse Ig reagents used in this study have been described in detail elsewhere. Briefly, antisera raised in goats after immunization with appropriate BALB/c myeloma proteins were made isotype specific by absorption with heterologous myeloma proteins. Antiallotype antibodies have been found in goat anti-mouse Ig antisera (17, 18) and have been shown to introduce significant error in measurements of isotypes of different allotypes (18). Because antiallotype antibodies directed against Ig^- (BALB/c) specificities were present in the antisera reactive with IgG1, IgG2a, and IgG2b, these reagents were prepared by acid elution from an immunoadsorbent carrying Ig^- (C57BL/6J) or Ig^- (A/J) Ig. This step resulted in the isolation of antibody reacting with isotypic specificities and possibly allotypic specificities common to Ig molecules of the Ig^-, Ig^-b, and Ig^-h phenotypes. The isotype specificity of these reagents was confirmed in a radioimmunoassay employing Sepharose beads to which myeloma proteins representative of each mouse Ig isotype (IgM, IgA, IgG1, IgG2a, IgG2b, and IgG3) had been coupled. Absorption studies showed that the anti-Ig reagents used in this study do not discriminate among Ig molecules from BALB/c (Ig^-), C57BL/6J (Ig^-b) or A/J (Ig^-h) mice and are therefore acceptable for comparative measurements of Ig molecules of the Ig^-, Ig^-b, and Ig^-h phenotypes. Because Ig^- and Ig^-b allotypes differ only at CH alleles coding for IgG2a, our reagents are suitable for comparative measurements of IgG1, IgG2a, and IgG2b molecules from CBA/Tufts (Ig^-b) and CBA.Igh^-b mice. Furthermore, our anti-IgG2a reagent does not discriminate between Ig^-b and Ig^-h IgG2a molecules. On this basis, we conclude that our comparisons of the levels of anti-SRBC antibody present in sera from CBA/Tufts (Ig^-b) and CBA.Igh^-b mice are valid.

Results

To What Extent is the Response to SRBC T Dependent? On day 14 after a primary immunization, nude mice produced <10% of the normal amounts of γs, γ1, γ2a, and γ2a anti-SRBC. They did produce ~25% of the normal IgM response (Fig. 1). Similar results (data not shown) were obtained on day 5 of a primary response and after multiple immunizations. Nude mice given 1 × 10^7 - 2 × 10^7 T cells produce normal

2 Haber, J., and H. Winn. An isotypic antiglobulin test for quantitation of anti-sheep red blood cell antibodies of individual Ig classes in mice bearing different IgCH haplotypes. Manuscript submitted for publication.
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Response as a % of normal

| isotype | 25 | 50 | 75 | 100 |
|---------|----|----|----|-----|
| μ       |    |    |    |     |
| τ3      |    |    |    |     |
| τ1      |    |    |    |     |
| τ2a     |    |    |    |     |
| τ2b     |    |    |    |     |

units of Ab X 10^-2/ml

6.2
0.6
0.6
0.3
0.8

Fig. 1. Serum levels of anti-SRBC found in nude mice 14 d after a primary immunization with 4 × 10^7 SRBC i.p. Values represent those found in sera pooled from six mice. A similar pool of sera from normal littermate mice was also analyzed. For details of the radioimmunoassay used to determine antibody (Ab) levels, see Materials and Methods.

| expt | isotype | 25 | 50 | 75 | 100 |
|------|---------|----|----|----|-----|
| 2    | μ       |    |    |    |     |
|      | τ3      |    |    |    |     |
|      | τ1      |    |    |    |     |
|      | τ2a     |    |    |    |     |
|      | τ2b     |    |    |    |     |

Response as a % of Ighj

A

| units of Ab X 10^-2/ml |
|------------------------|
| 11.7                   |
| 2.4                    |
| 200                    |
| 2.6                    |
| 121                    |
| 2.3                    |
| 230                    |
| 6.2                    |

B

| units of Ab X 10^-2/ml |
|------------------------|
| 21.4                   |
| 3.5                    |
| 220                    |
| 24.4                   |
| 10.8                   |
| 30.8                   |

Fig. 2. Serum levels of anti-SRBC found in CBA/Igh^b mice 7 d after a primary (A) or secondary (B) immunization with 4 × 10^7 SRBC i.p. Values represent those found in sera pooled from six mice. A similar pool of sera from age- and sex-matched CBA/Tufts (Igh^j) mice was also analyzed. Ab, antibody; (--) SEM.

responses to SRBC (Fig. 3 A, below). Thus for all five isotypes examined the majority of the anti-SRBC response is T dependent.

Do Igh^j and Igh^b Mice Produce Different Amounts of Anti-SRBC Antibody? Because we were interested in comparing the helper ability of T cells from Igh^j and Igh^b, mice it was important to establish that the donor strains do not have quantitatively different responses to SRBC. As shown in Fig. 2, after either a primary or a secondary immunization the mice from the two strains produced comparable amounts of anti-SRBC of each isotype. Therefore, there was no reason to believe that the presence of
either the Igh^a or the Igh^b haplotype in itself produced an enhanced anti-SRBC response.

Do the Igh^b Haplotypes Restrict the Ability of T Cells to Generate Help for Igh^b B Cells in either a Primary or a Secondary Response? Peripheral T cells from unprimed donors, either Igh^a or Igh^b, were transferred to Igh^a nude hosts and the host animals immunized. The presence of T cells of both genotypes provided help for nude Igh^b B cells. For each isotype examined the amount of antibody found on day 12–13 was similar regardless of the T cell genotype. (Fig. 3A). Therefore the presence of an Igh^b genotype (or the absence of the Igh^a genotype) does not prevent T cells from providing help for Igh^a B cells. This result was confirmed when the recipient animals were challenged on day 21 (Fig. 3C).

Are T Cells from SRBC-immunized Igh^a and Igh^b Mice also Equivalent in Providing Help for Igh^b B Cells? In contrast to the previous results T cells from SRBC-primed Igh^a donors failed to provide as much help as Igh^a T cells (Fig. 3C). On subsequent challenge, these Igh haplotype mismatched T cells were also less efficient than their matched counterparts in generating help (Fig. 3D).

Is Priming of TH Influenced by the Igh Phenotype of the Environment? An examination of the amount of antibody produced in the course of a primary response to SRBC indicates that Igh^b T cells from both primed and unprimed donors provide as much help as unprimed Igh^a T cells but far less help than T cells from SRBC-primed Igh^a donors (Table I). That is, whereas Igh^b mice can provide helper T cells, they do not
show any evidence that they can be primed by SRBC to provide augmented help for Igh\(^b\) B cells. This result contrasts with that seen with Igh\(^j\) donor mice where priming with 4 × 10\(^6\) SRBC i.p. 6 d before T cell transfer resulted in a marked augmentation of help for all isotypes. This means that either contact with SRBC induced different levels of help in Igh\(^j\) and Igh\(^b\) T cells or that Igh\(^j\) and Igh\(^b\)-linked genes produce a difference in the priming environment that effects the helper capability of T cells from the two strains.

The first conclusion is unlikely because (a) the primary and secondary responses of the two strains are similar (Fig. 2); and (b) the responses to a secondary immunization of Igh\(^j\) mice given either unprimed Igh\(^j\) or Igh\(^b\) T cells were equivalent (Fig. 3 B). That is, T cells of the Igh\(^b\) genotype were primed to help Igh\(^j\) B cells provided that Igh\(^j\)-linked products were present at the time of SRBC immunization (Table II).

A more direct test of this last conclusion was possible using a double transfer of T cells. T cells of either Igh\(^j\) or Igh\(^b\) genotype were transferred to Igh\(^j\) nude mice which were then immunized with 4 × 10\(^6\) SRBC. 6 d later, T cells from these primary recipients were recovered and transferred to second group of Igh\(^j\) nudes. Although Igh\(^b\) T cells from intact Igh\(^b\) SRBC-primed mice failed to provide any help when
The central observation of these experiments is that the ability of T cells to help in the generation of an antibody response is strongly influenced by Igh-linked genes of the cellular environment. This observation rests on the demonstration that the majority of antibody to SRBC—IgM, IgG3, IgG1, IgG2b, and IgG2a—is T cell dependent as demonstrated by the weak responses of nude mice (Fig. 1) and the vigorous responses of T cell-restored nudes (Fig. 3). This confirms previous findings (19), as does our observation that IgG responses are more T dependent than IgM responses (20). That some antibody, mainly IgM but also IgG, appears in nude mice could mean either that there is a T-independent response to some determinants and/or that there is sufficient (weak) T help in the nude (21) to permit these small responses.

We did not find any significant difference in the amounts of antibody produced by Igh\(^a\) and Igh\(^b\) mice. This could mean either that the T cells of the two strains provide equivalent amounts of help for their own B cells, or that the T cells of one strain provide less help but that their B cells make a more vigorous response. The first explanation is probably correct because when T cells from either strain were allowed to collaborate with Igh\(^b\) B cells, the amounts of antibody generated were equivalent (Fig. 3 A, and Table I). This equivalence continues through a second immunization.

| Expts. | Isotype | 25  | 50  | 75  | 100 |
|--------|---------|-----|-----|-----|-----|
| 5,12   | \(\gamma_3\) | 254 |     |     |     |
| Day 28 (7) | \(\gamma_1\) |     |     |     | 193 |
| 4,8,10,11 | \(\gamma_2a\) |     |     |     | 14  |
| Day 28 (7) | \(\gamma_2b\) |     |     |     |     |

Transferred to Igh\(^d\) nudes, Igh\(^b\) T cells that were in an Igh\(^d\) environment at the time of SRBC priming provided help (Fig. 4).

### Discussion

The central observation of these experiments is that the ability of T cells to help in the generation of an antibody response is strongly influenced by Igh-linked genes of the cellular environment. This observation rests on the demonstration that the majority of antibody to SRBC—IgM, IgG3, IgG1, IgG2b, and IgG2a—is T cell dependent as demonstrated by the weak responses of nude mice (Fig. 1) and the vigorous responses of T cell-restored nudes (Fig. 3). This confirms previous findings (19), as does our observation that IgG responses are more T dependent than IgM responses (20). That some antibody, mainly IgM but also IgG, appears in nude mice could mean either that there is a T-independent response to some determinants and/or that there is sufficient (weak) T help in the nude (21) to permit these small responses.

We did not find any significant difference in the amounts of antibody produced by Igh\(^b\) and Igh\(^b\) mice. This could mean either that the T cells of the two strains provide equivalent amounts of help for their own B cells, or that the T cells of one strain provide less help but that their B cells make a more vigorous response. The first explanation is probably correct because when T cells from either strain were allowed to collaborate with Igh\(^b\) B cells, the amounts of antibody generated were equivalent (Fig. 3 A, and Table I). This equivalence continues through a second immunization.
with SRBC, a finding indicating that T cells of both genotypes can provide help for B cell clonal expansion and triggering. This conclusion rests on the observation that both the establishment of B cell memory, and its expression requires interaction with T cells (22). In summary we can say that Igh\textsuperscript{b}- and Igh\textsuperscript{a}-linked genes do not limit the potential for T and B cells to collaborate.

In contrast, T cells introduced to SRBC in an environment that does not contain the products of Igh\textsuperscript{a}linked genes are inefficient helpers for Igh\textsuperscript{b} B cells. (Figs. 3 C and D and Tables I and II). In terms used in other contexts, these T cells are restricted or adaptively differentiated with respect to the products of Igh-linked genes.

Our interpretation of this result is that B cells are triggered by antigen (SRBC) and produce SRBC-specific, Ig-containing products. These products serve to trigger a set of TH bearing receptors that are complementary to the Ig products. As a result of proliferation and differentiation, an expanded pool of Ig-specific memory T cells is produced. These primed cells then serve as effector T helper cells with specificity for B cells bearing molecules structurally similar to those produced by B cells that originally responded to SRBC.

Although we favor the idea that B cells are the source of the priming Ig product, we have not ruled out the possibility that other cells, e.g. cells of the T lineage (which would be present in nude mice [23-25]) are responsible. Direct evidence implicating B cells is provided by L'age-Stehr (26).

Because it requires antigen to generate a B cell product that is coded for by Igh-linked genes, it is reasonable to propose, as we do, that the B cell product contains the immunoglobulin heavy chain. However, we can not, with certainty, rule out the
possibility that the products of other loci, e.g., minor histocompatibility loci, are the relevant molecules. We favor the idea that there are Ig-specific helper cells because such cells have been shown to exist in three entirely different helper systems: isotype (10), allotype (7), and idiotype (8, 9). This means that two classes of TH have been demonstrated: carrier specific and Ig specific. Independent estimates of the number of interacting T cells have also suggested the evidence of two populations (27). In fact, there is some evidence at hand that one of these populations does depend on the presence of B cells with sIg (28). Proof of the Ig specificity of the helper T cell receptor requires a more direct test, such as the demonstration of the ability of these T cells to specifically bind (anti-SRBC) antibody.

Assuming that we are seeing help provided by T cells with anti-Ig specificity, we can not respond to the question as to whether these cells recognize V_H or C_H sequences. Resolution of this point requires experiments using V_H-C_H recombinant mice (26) and appropriate T cell-Ig-binding studies. Although the demonstration of allotype-, isotype-, and idiotype-specific help is consistent with the possibility that TH_Ig populations exist that have receptors for each of these three specificities, it is not a proof. The nonrandom association of V_H products with C_H products (29) would lead to allotype- or isotype-specific help even if TH_Ig had receptors only for V_H sequences.

If, indeed, there are TH_Ig, we do not know if they are specifically stimulated by free antibody, surface antibody, or antigen-antibody complexes. Nor do we know if the responding T cells must bind other molecules (e.g., antigen or H-2 product) to be effectively triggered. The possibility exists, for instance, that TH_ear are Ia restricted with respect to antigen-presenting cells, whereas TH_Ig are (Ia?) restricted by the phenotype of the B cell.

Although T cells from SRBC-primed Igh_b mice were less helpful than syngeneic cells they did provide substantial help. Does this help represent TH_Ig and/or TH_ear help? At this time we can not say. It is possible that all the allogeneic help is a result of TH_ear. Alternatively, there could be some cross-reactivity between Igh_b and Igh_l determinants that are recognized by TH_Ig, or in the course of their residence in Igh_l nudes, the Igh_b TH_Ig are primed by B cell products from their hosts. The nature of the interaction between help provided by TH_ear and TH_Ig awaits future exploration (9, 30, 31).

Summary

The role of Igh-linked loci in the generation and expression of T cell help for antibody responses to sheep erythrocytes (SRBC) was investigated. The production of IgM, IgG3, IgG1, IgG2a, and IgG2b antibody to SRBC was shown to be T cell dependent. The Igh-congenic mouse pair CBA/Tufts (Igh_l) and CBA.Igh_b gave equivalent responses to SRBC. CBA.nude mice (Igh_l) supplemented with peripheral T cells of either Igh_l or Igh_b genotype produced equivalent, high responses. Therefore, T cell-B cell mismatching for the Igh haplotype is not in itself a bar to the generation or expression of help. In contrast, T cells primed in an environment that lacks Igh_l-linked products are inefficient helpers for Igh_l B cells. These results suggest that antigen-primed B cells or their products prime a set of T cells that can help B cells that bear matching, Igh-linked gene products.

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