

# Maintenance of antigen-specific immunological memory through variable regions of heavy and light chains of anti-idiotypic antibody

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## Summary

Immunological memory is characterized by a quick and enhanced immune response after re-exposure to the same antigen. To explain the mechanism involved in generation and maintenance of immunological memory, we had earlier proposed a hypothesis involving the relay of memory by idiotypic and anti-idiotypic B cells. The peptidomimic present in the hypervariable region of anti-idiotypic antibody was hypothesized to carry forward immunological memory. In the present work, we provide evidence supporting a role for the anti-idiotypic antibody in eliciting antigen-specific B-cell and T-cell responses. Employing the idiotypic monoclonal antibody ( $Ab_1$ ) specific for haemagglutinin (H) protein of rinderpest virus,  $Ab_{2\beta}$  was generated, which possesses an internal image of the H protein in the region between amino acids 527 and 556. We demonstrate that antigen-specific memory is perpetuated by immunization with  $Ab_2$ , as shown by maintenance of antigen-specific T-cell responses upon restimulation *in vitro* of  $Ab_2$  immune splenocytes by antigen-presenting cells expressing H protein or pulsed with H-protein-derived peptides. We have also shown that boosting with antigen-specific anti-idiotypic B cells generates a memory response in antigen-primed mice. Evidence has been provided for the existence of an antigen-specific B-cell idiotypic network in the body that supports the perpetuation of immunological memory as proposed in the relay hypothesis.

**Keywords:** anti-idiotypic-B cells; idiopeptide; immunological memory; peptidomimics; relay hypothesis

## Introduction

Immunological memory is an intrinsic property of the immune system. Factors governing the generation and perpetuation of immunological memory have been the subject of many investigations but these have not described any clear-cut mechanism to account for its perpetuation. The immune system of an individual can make millions of different kinds of antibodies. Each antibody can in turn be the target of other antibodies that recognize its unique molecular determinants. Idiotypes (sets of idiotopes) are determinants expressed in the variable

region of the antibody molecules and T-cell receptors. Jerne's Network theory predicts that idiotypic and anti-idiotypic interactions constitute an immune network that is involved in the regulation of the immune responses.<sup>1</sup>

A mechanism has been proposed<sup>2</sup> that explains the perpetuation of B-cell immunological memory indefinitely without requiring the presence of long-living memory cells or persisting antigen. The hypothesis combines the essential features of Burnet's clonal selection theory and Jerne's network hypothesis and considers both B-cell and T-cell memory. According to this hypothesis, the key event for generation of a memory response is the

Abbreviations: Ab, antibody; APC, antigen-presenting cell; APL, altered peptide ligand; BCR, B-cell receptor; CDR, complementarity-determining region; CTL, cytotoxic T lymphocyte; DC, dendritic cell; ELISA, enzyme-linked immunosorbent assay; H protein, haemagglutinin protein; Id, idiotype; IFN- $\gamma$ , interferon- $\gamma$ ; Ig, immunoglobulin; IL, interleukin; MHC, major histocompatibility complex; OD, optical density;  $V_H/V_L$ , variable region of heavy/light chains of immunoglobulin.

interaction of idiotypic (antigen-specific cells or Burnet cells) and anti-idiotypic cells (cells that are reactive to idiotype determinant or Jerne cells). These idiotype and anti-idiotype B cells mutually stimulate and clonally expand, with either specific or bystander T-cell help. The anti-idiotypic cells carry a peptidomimic, which drives the memory response further by triggering idiotypic memory T cells. Because B cells can present antigen,<sup>3</sup> they present 'apparently foreign' idiopeptides to T cells. The idiopeptides of *de novo* synthesized antibody are presented to CD8<sup>+</sup> T cells through class I major histocompatibility complex (MHC), which recognizes the idiopeptide-presenting cells as targets and regulates their population. The recycling of immunoglobulins from the surface to the endosomal compartment of B cells leads to the presentation of idiopeptides to CD4<sup>+</sup> T cells by class II MHC. Even if the majority of the clonally expanded cells die as a result of lack of stimulation, cytotoxic T-lymphocyte (CTL) lysis or for other reasons, the surviving cells are able to carry forward the memory. This mechanism also provides a means for affinity maturation through the idiotype selection of somatically mutated high-affinity cells or those from the naive pool.

There is experimental evidence for the relay hypothesis described above, and it has been shown that the idiotype and anti-idiotypic B cells are generated in the same animal after immunization with antigen<sup>4</sup> and that T cells are involved in the idiotype-anti-idiotype B-cell network.<sup>5</sup> Anti-idiotypic B cells, which carry peptidomimic in their antigenic determinants, are thought to play a crucial role in the maintenance and regulation of B-cell and T-cell memory, as originally proposed in the relay hypothesis. A role for serum immunoglobulins in the perpetuation of immunological memory has also been proposed.<sup>6</sup>

The immune system is a functional idiotype network and anti-idiotypic antibodies are components of the normal immune system.<sup>7-10</sup> It has been proposed that one of the peripheral regulatory mechanisms involves recognition of internal image by the idiotype determinants of specific antibodies or T lymphocytes, which regulate the immune response to both foreign and self-antigens.<sup>1,2,11</sup> Anti-idiotypes have long been used as priming agents or sole immunogens, in conjunction with antigen or coupled with tetanus toxoid, interleukin-2 (IL-2) or granulocyte-macrophage colony-stimulating factor,<sup>12,13</sup> for the production of antibodies reactive to virus, bacteria or tumour antigens. It has also been shown that part of transplacental immunoglobulin G (IgG) antibodies also contain anti-idiotypic antibodies that might prime the immune system of the offspring.<sup>14</sup> However, the role of anti-idiotypic antibodies in the long-term perpetuation of antigen-specific immunological memory in the absence of an antigenic stimulus has not been established.

In the present work, we describe the generation and characterization of syngeneic monoclonal anti-idiotypic

antibodies against a monoclonal antibody that recognizes the envelope glycoprotein haemagglutinin (H) of rinderpest virus. The main purpose of this study was the generation and analysis of the internal image of H protein in the form of an anti-idiotypic antibody, which may have the potential to elicit virus-specific immune responses and may maintain the immunological memory. Anti-idiotypic monoclonal antibody D9D8 (Ab<sub>2</sub>) was produced against a monoclonal idiotype antibody A12A9 (Ab<sub>1</sub>, which had been generated earlier)<sup>4</sup> for which the antigenic site on H protein lies between amino acid residues 527 and 556. We show that mimicry of the H antigen by anti-idiotypic antibody D9D8 is associated with a 12-amino-acid sequence on its heavy-chain hypervariable region. This sequence is partially homologous with the epitope on H protein, which is conserved in the H protein of viruses in the morbillivirus genus. The anti-idiotypic antibody was able to elicit antigen-specific B-cell and T-cell responses when given as soluble or cell-bound protein. In addition, the boosting of antigen-primed animals with anti-idiotypic B cells generates antigen-specific memory B-cell and T-cell responses. We demonstrate that an idiotype-anti-idiotypic network of antigen-specific B cells, which also involves T cells, exists in the body of immunized animals. This network is capable of relaying the memory in the absence of antigen to succeeding generations of B and T cells.

## Materials and methods

### Animals

BALB/c mice and Sprague Dawley rats were bred and maintained in the Central Animal Facility, Indian Institute of Science, Bangalore, in accordance with the institutional guidelines. Female mice, 8-12 weeks old, were used in all experiments.

### Cell lines

A12A9 (Ab<sub>1</sub>), a B-cell hybridoma cell line of BALB/c origin, was generated against the purified recombinant haemagglutinin protein of rinderpest virus.<sup>15</sup> The murine myeloma cell line sp2/0 and, the hybridoma cell line A12A9 (Ab<sub>1</sub>) and Ab<sub>2</sub> cell line (D9D8) generated in this work were maintained in Iscove's modified Dulbecco's medium supplemented with 10% heat inactivated fetal bovine serum (FBS) (Life Technologies, Grand Island, NY), 2 mM/l L-glutamine, 100 U/ml penicillin (Gibco-BRL, New York, NY), and 100 µg/ml streptomycin (Gibco). P815 (H-2<sup>d</sup>) murine mastocytoma cells were cultured in RPMI-1640 medium (Gibco-BRL) supplemented with 5% FBS (Life Technologies). Sf-21 cells were maintained in TC-100 medium (Gibco-BRL) supplemented with 10% FBS (Life Technologies). Baculovirus expressing

full-length H protein as secretory protein<sup>16</sup> was used to infect these cells at a multiplicity of infection of 20 and the secreted H protein was collected from the medium and purified by immunoaffinity chromatography. The purified H protein was used as a source of antigen.

#### *Cytokines*

All cytokine standards and cytokine-specific antibodies were purchased from E-bioscience (San Diego, CA). Recombinant murine IL-4 was purchased from Pharmin-gen (San Diego, CA).

#### *Peptides*

Peptides were designed based on the amino acid sequence homology of D9D8 and H protein. They were procured from Peptron (Daejeon, South Korea). The purity of peptides was > 90% by high-performance liquid chromatography analysis. The sequences of the peptides were YYYPFKLPI (H-1), IYSTTGRLS (H-2), GSTYYPDSV (V<sub>H</sub>), RLLIYLVSL (V<sub>L</sub>).

#### *Immunization and hybridoma production*

Three female 8-week-old BALB/c mice were injected subcutaneously with monoclonal anti-haemagglutinin hybridoma cells (secreting A12A9 antibody;  $20 \times 10^6$ ) irradiated at 3500 rads and boosted twice with the same number of cells at a 1-week interval. Sera collected from the immune mice were tested for the presence of anti-idiotypic antibody. Hybridoma cells were generated by polyethyleneglycol-mediated fusion of immune splenocytes with Sp2/0 cells followed by two cycles of sub-cloning by limiting dilution.<sup>17</sup>

#### *Detection of anti-idiotypic antibody*

The anti-idiotypic antibody was detected by enzyme-linked immunosorbent assay (ELISA) employing purified polyclonal anti-H antibody (200 µg/well) raised in rabbit. The presence of anti-idiotypic antibody in culture supernatants was also monitored by FACScan flow cytometry (B&D, San Diego, CA) using monoclonal Ab<sub>1</sub> cells bearing surface immunoglobulin and anti-mouse IgG fluorescein isothiocyanate conjugate. The clone D9D8, which consistently produced high titre antibodies as determined by ELISA and fluorescence-activated cell sorting, was chosen for further experiments and the isotype of this clone was determined using an Isotype ELISA kit (Boehringer-Mannheim, GmbH, Mannheim, Germany) following the kit protocol. IgG for D9D8 was purified from ascitic fluid by affinity chromatography on a protein A-Sepharose 4B column (Pharmacia, Uppsala, Sweden). The purity of the isolated immunoglobulin (> 99%) was

determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis.

#### *Generation of anti-anti-idiotypic antibody (Ab<sub>3</sub>)*

Sprague Dawley female rats (12 weeks old) were immunized by two intraperitoneal injections of purified D9D8 (200 µg) in complete Freund's adjuvant with a 1-week interval. After 2 weeks, sera were collected and booster was given with the same amount of antigen in incomplete Freund's adjuvant. The 2-week post-booster serum was checked for the presence of anti-anti-idiotypic antibody by ELISA.

#### *Competitive ELISA*

Purified Ab<sub>1</sub> was biotinylated using ImmunoProbe™ Biotinylation Kit (Sigma, St. Louis, MO) following the manufacturer's instructions. Microtitre plates (high-binding, Costar, Cambridge, MA) were coated with 200 µg H protein, blocked with 2% bovine serum albumin, reacted with a mixture of 50 µl biotinylated Ab<sub>1</sub> (A12A9) (1 : 1000 dilution) and 50 µl varying concentrations Ab<sub>2</sub> (10 µg to 0.01 µg) or control anti-bodies (normal mouse IgG and purified X2A5, an isotype-matched non-specific antibody). Following a 1-hr incubation, avidin-horseradish peroxidase was added and colour was developed by addition of H<sub>2</sub>O<sub>2</sub> and *o*-phenylenediamine. The per cent inhibition by Ab<sub>2</sub> of the binding of Ab<sub>1</sub> to antigen was calculated by taking the optical density of the well containing no competitor as 100%.

#### *Polymerase chain reaction amplification and sequence analysis of variable region of heavy and light chains*

Cytoplasmic RNA was extracted from D9D8 hybridoma cells ( $3 \times 10^6$  cells) by the TRIZOL method. Reverse transcription and polymerase chain reactions were performed. Variable regions of the heavy (V<sub>H</sub>) and light (V<sub>L</sub>) chains of immunoglobulin were amplified in two different reactions using IgG-specific primers (forward primer: TGAG GAGACGGTGACCGTGGTCCCTTGGCCCC; reverse primer: AGGTSMARCTGCAGSAGTCWGG) and κ-chain-specific primers (forward primer: GACATTGAGCT CACCCAGTCTCN; reverse primer: CTCGAGTTTGAGT TCCAGCTTN), respectively. Polymerase chain reaction products were subsequently cloned into pGEM-T EASY (Promega, Madison, WI) vector and were sequenced by standard DNA sequencing techniques. The amino acid sequence of V<sub>H</sub> and V<sub>L</sub> was deduced from the nucleotide sequence using a DNA-translate tool (<http://www.expasy.org/tools/dna.html>). The V<sub>H</sub> and V<sub>L</sub> sequences were aligned with the known sequence of H antigen using the Global Alignment Program (GAP) algorithm<sup>18</sup> to check for homologous regions.

### Generation of a P815 cell line stably expressing H protein

P815 cells were plated in 60-mm tissue culture Petri plates and transfected with 10 µg H-pFLAG-CMV-3 plasmid DNA (Sigma) (harbouring a full-length H gene subcloned from the pBluescript (PBS) vector<sup>16</sup> into eukaryotic pFLAG cytoplasmic expression vector) in serum-free medium using lipofectamine (Life Technologies). Stable cell lines were made following the protocol mentioned in Nilsson and Nilsson.<sup>19</sup> Positive clones (P815-H cells) were maintained in complete medium containing 400 µg/ml of G418 (Calbiochem, LaJolla, CA). Protein expression in the cells was confirmed by fluorescence microscopy, sodium dodecyl sulphate–polyacrylamide gel electrophoresis and enhanced chemiluminescence Western blotting of the cell lysates using H-protein-specific rabbit polyclonal antibody and anti-flag antibody.

### Lymphocyte proliferation assay

Responder T cells (T cells from H-protein-immune and Ab<sub>2</sub>-immune mice) were isolated as described earlier<sup>20</sup> and were plated at a density of  $5 \times 10^5$  cells/well in 96-well flat-bottomed tissue culture plates in the presence of either P815-H cells, purified H protein, soluble Ab<sub>2</sub> or P815 cells pulsed with synthetic peptides derived from the mimic regions of H protein, V<sub>H</sub> or V<sub>L</sub>. Anti-rinderpest virus N-protein-specific monoclonal antibody and purified rinderpest virus P protein were used as negative controls. Cells were cocultured for 72 hr and then pulsed for 16 hr with [<sup>3</sup>H]thymidine (1 µCi/well). Cells were harvested on glass fibre filters using a semi-automated cell harvester (Nunc, Roskilde, Denmark). [<sup>3</sup>H]Thymidine incorporation was measured in a liquid scintillation spectrophotometer and T-cell proliferation was measured as mean counts/min of triplicate wells. For recall response experiments, lymphocytes isolated from H-protein-immune and differentially boosted mice were restimulated *in vitro* with purified H protein (50 µg/ml) and a proliferation assay was carried out as described above.

### Cytotoxic T-cell assay

Single cell suspensions from lymph nodes isolated from H-protein-immune and differentially boosted mice were seeded in complete medium at a concentration of  $2 \times 10^6$  cells/well of 24-well plates. P815-H cells ( $1 \times 10^6$  cells/well) irradiated at 4000 rads were used as antigen-presenting cells. The cells were cocultured for 10 days in the presence of antigen-presenting cells and IL-2 (100 units/ml). Viable cells were harvested and were used as effector cells in a CTL assay. Antigen-specific CTL activity was assessed employing P815-H cells as targets at different effector : target ratios. CTL activity was measured with a cytotoxic assay kit (Roche, Mannheim, Germany) following the manufacturer's instructions.

### Cytokine ELISA

Splenocytes from the immune mice were incubated with various restimulants for 72 hr as described above for cell proliferation assays. Supernatants from the culture were collected for cytokine assays. IL-2, IL-4 and interferon-γ (IFN-γ) were assayed using cytokine ELISA kits (e-Bioscience).

### Intracellular cytokine staining

Lymph node cells from H-protein-immunized mice and P815-H cells were cocultured for 4 hr. Brefeldin-A was added at a final concentration of 10 µg/ml 6 hr later, cells were harvested and after permeabilization with 0.5% saponin, intracellular staining was performed using anti-mouse IFN-γ–biotin-conjugated antibody and streptavidin–phycoerythrin (Pharmingen).

## Results

### Anti-idiotypic antibody response in mice

There were 180 clones as a result of the fusion of Sp2/0 myeloma cells and splenocytes from BALB/c mouse immunized with Ab<sub>1</sub> (A12A9), of which 14 clones produced antibodies that reacted with Ab<sub>1</sub>. Two clones (D9D8 and D9E6) secreting antibodies that reacted best with Ab<sub>1</sub> both in ELISA and flow cytometry were selected for further study. Both produced antibodies of IgG heavy and κ light chain isotypes. The D9D8 hybridoma was selected for all further studies.

### Anti-anti-idiotypic antibody response

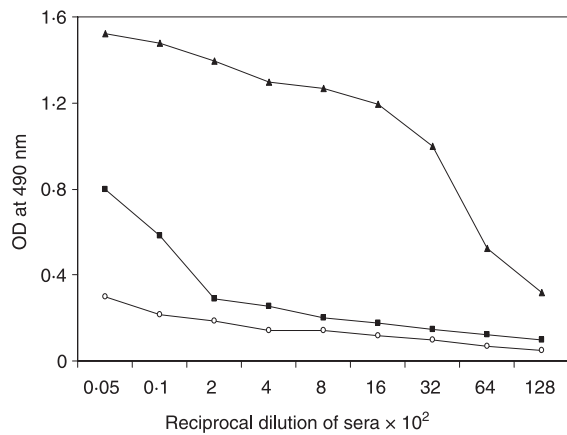
To test whether D9D8 Ab<sub>2</sub> is an antigen mimic (Ab<sub>2β</sub>), purified Ab<sub>2</sub> protein was used to immunize rats. The reactivity of rat serum with antigen H was measured in ELISA. The results illustrated in Fig. 1 show that Ab<sub>3</sub> made in rats reacts with the antigen and thus is similar to Ab<sub>1</sub>. Thus, Ab<sub>2</sub> D9D8 induces Ab<sub>3</sub>, which recognizes the original antigen H, indicating that D9D8 is Ab<sub>2β</sub>.

### Ab<sub>2</sub> recognizes Ab<sub>1</sub> from different animal species

D9D8 (Ab<sub>2</sub>) recognized antibodies made in different animal species against rinderpest virus infection or H protein immunization (Table 1). These results confirmed that Ab<sub>2</sub> is indeed a functional H protein mimic.

### Anti-idiotypic antibody competes with H protein for binding to idiotypic antibody

Ab<sub>2</sub> (D9D8) inhibited the binding of Ab<sub>1</sub> to antigen in a dose-dependent manner (Fig. 2). D9D8 at a concentration



**Figure 1.** Determination of D9D8 as Ab<sub>2β</sub> by ELISA. Plates were coated with purified recombinant H protein (200 ng/well) and incubated with rat sera (pre-immune, ○; pre-boost, ■ and post-boost, ▲) raised against purified Ab<sub>2</sub> at different dilutions. Donkey anti-rat-horseradish peroxidase was used as the secondary antibody. Data shown are the average optical density (OD) of triplicate wells.

**Table 1.** Reactivity of purified Ab<sub>2</sub> (D9D8) with heterologous sera as the source of Ab<sub>1</sub><sup>1</sup>

Immunizing agent	Species	Titre of Ab <sub>1</sub> in immune in sera <sup>2</sup>
Recombinant Baculo H protein (soluble)	Cattle	6.4
H protein displayed on recombinant extracellular baculovirus	Cattle	> 3.2
Recombinant baculo H protein (membrane-bound form)	Rabbit	3.2
Peste des petits ruminants virus	Goat <sup>3</sup>	> 6.4
Rinderpest virus	Cattle <sup>4</sup>	> 3.2

<sup>1</sup>Microtitre plates were coated with 200 ng/well of Ab<sub>2</sub> and dilutions of H-specific immune sera from different sources was added.

<sup>2</sup>Data shown are titre (× 10<sup>3</sup>) of Ab<sub>1</sub> recognized by Ab<sub>2</sub> and are calculated from average optical density (OD) of triplicate wells on comparison with OD of respective dilutions of pre-immune sera. Normal rabbit, bovine and goat serum were used as controls and the OD values of controls were subtracted from the experimental values.

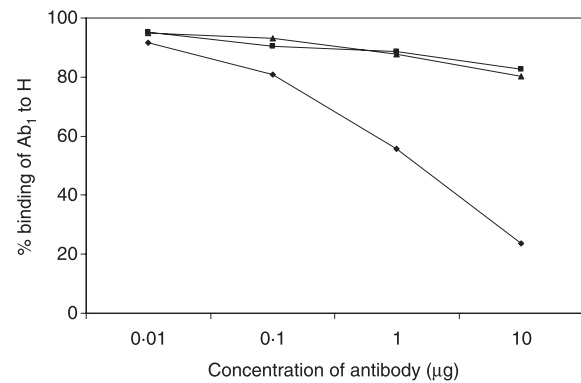
<sup>3</sup>Hyper-immunized goats.

<sup>4</sup>Convalescent cattle.

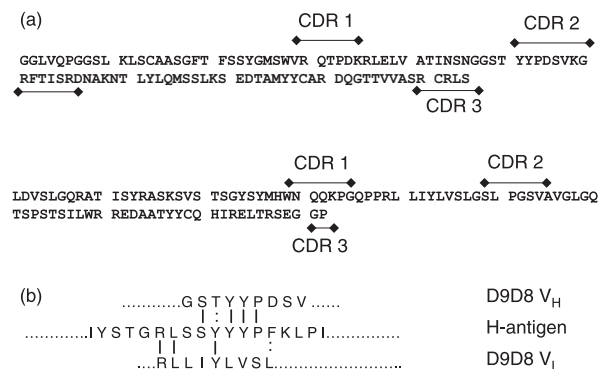
of 10 μg/ml inhibited the binding of A12A9 to H protein by 76% while the control antibodies did not cause any significant inhibition over a range of concentrations. This indicated that D9D8 has the functional epitope for Ab<sub>1</sub>, which is similar to the epitope on H protein.

### Sequence analysis of anti-idiotypic antibody

The sequences of V<sub>H</sub> and V<sub>L</sub> are given in Fig. 3(a). Hypervariable regions (CDRs) and framework regions

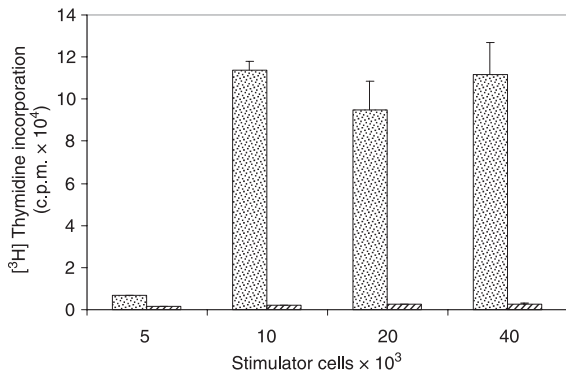


**Figure 2.** Inhibition of Ab<sub>1</sub> (A12A9) binding to Sec H by Ab<sub>2</sub> (D9D8) in ELISA. Sec H protein 100 ng/well was the coating antigen. Inhibition of binding of biotinylated A12A9 to Sec H was tested in the presence of different concentrations of D9D8 (◆). Normal mouse IgG (●) and X2A5 (▲), an isotype-matched control antibody (purified) raised against rinderpest virus nucleocapsid protein, were used as controls at the same concentrations. Data represent average optical density (OD) of triplicate wells.



**Figure 3.** (a) Sequences of variable regions of heavy (V<sub>H</sub>) and light (V<sub>L</sub>) chains of D9D8. (b) Alignment of homology regions between V<sub>H</sub>/V<sub>L</sub> sequences and antigenic region carrying the epitope on rinderpest virus H protein showing the 'Internal Image'.

(FRs) on these sequences were located using immunoglobulin BLAST of NCBI (<http://www.ncbi.nlm.nih.gov/igblast/>). The epitopic site on H protein for Ab<sub>1</sub> was mapped to the region between amino acids 525 and 556 employing deletion mutants of H protein. Alignment of the Sec-H-epitope-containing sequence with that of D9D8 (Fig. 3b) using the global alignment programme (GAP) indicated a significant homology with heavy chain CDR2. Fifty-two per cent similarity and 48% identity were seen between the epitopic region and CDR2 (amino acids 32–54) of the D9D8 V<sub>H</sub> (in a 15-amino-acid stretch, nine amino acids were identical and one was similar) while between V<sub>L</sub> and the epitopic region 35% similarity and 28% identity were seen, corresponding to part of the CDR2 and part of the FR2 regions (a two-amino-acid similarity and a one-amino-acid identity).

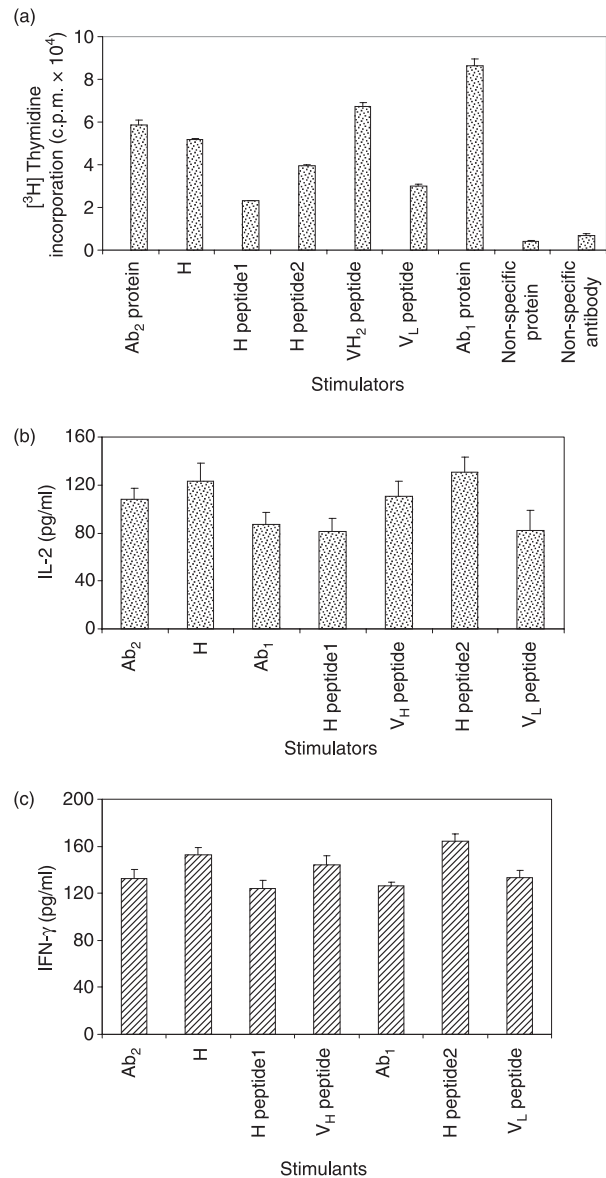


**Figure 4.** Proliferation of Ab<sub>2</sub> immune splenocytes ( $5 \times 10^5$  cells/well) on *in vitro* restimulation by P815 cells stably expressing H (stippled bars) and wild-type P815 (striped bars) cells. The data shown are the mean counts/min of triplicate wells.

### Anti-idiotypic Ab immunization generates an antigen-specific immune response

To test the generation of an antigen-specific T-cell response elicited by anti-idiotypic antibody, T-cell proliferation assays using T cells from anti-idiotypic-antibody-immune mice with various *in vitro* restimulants were performed. P815-H cells, which express H protein endogenously, were used as antigen-presenting cells and proliferation of immune T cells was monitored (Fig. 4). The proliferation was equally good, in response to soluble H, although the immune animals had never been exposed to the H protein. This response is 70% of the response generated when H-immunized splenocytes were restimulated with P815-H cells (data not shown). No cross-reaction was observed when immune cells were restimulated with irrelevant antibody or irrelevant protein. As can be seen from thymidine incorporation (Fig. 5a), the Ab<sub>2</sub> immune cell population had substantial levels of antigen-primed T cells by virtue of selective proliferation of T cells by the antigen mimic present on the anti-idiotypic B cells. The cells were also stimulated with synthetic peptides from the mimic regions on H and V<sub>H</sub> and V<sub>L</sub> of Ab<sub>2</sub>. Cells stimulated with the soluble H as well as H-derived synthetic peptide (IYSTTGRLS) exhibited greater proliferation, as shown by increased thymidine incorporation when compared to Ab<sub>2</sub>-stimulated cells. The presence of idiotype cascade was monitored using Ab<sub>1</sub> for *in vitro* restimulation, which also showed a substantial level of cell proliferation. Thus, it is evident that antigen-specific and idiotype-specific T cells are generated *in vivo* and coexist, probably as a result of the operation of an idiotype-anti-idiotypic network.

When cytokine patterns were evaluated, it was observed that *in vitro* stimulation of Ab<sub>2</sub> immune cells with soluble Ab<sub>2</sub> (Fig. 5b) produced high levels of IL-2 and IFN- $\gamma$ . H protein and H-2 peptide (IYSTTGRLS) restimulated cells



**Figure 5.** (a) Proliferation of Ab<sub>2</sub> immune splenocytes on *in vitro* restimulation with purified Ab<sub>1</sub>, H protein, H-specific synthetic peptides [H-1, H-2], V<sub>H</sub> peptide and V<sub>L</sub> peptide (10  $\mu\text{g/ml}$ ) derived from the homology regions. The data shown are the mean counts/min of triplicate wells. (b) IL-2 and IFN- $\gamma$  production by *in vitro* restimulated Ab<sub>2</sub> immune splenocytes in response to stimulation by full-length antigen and antigen-derived peptides (8  $\mu\text{g/ml}$ ). The results shown are representative of three independent experiments.

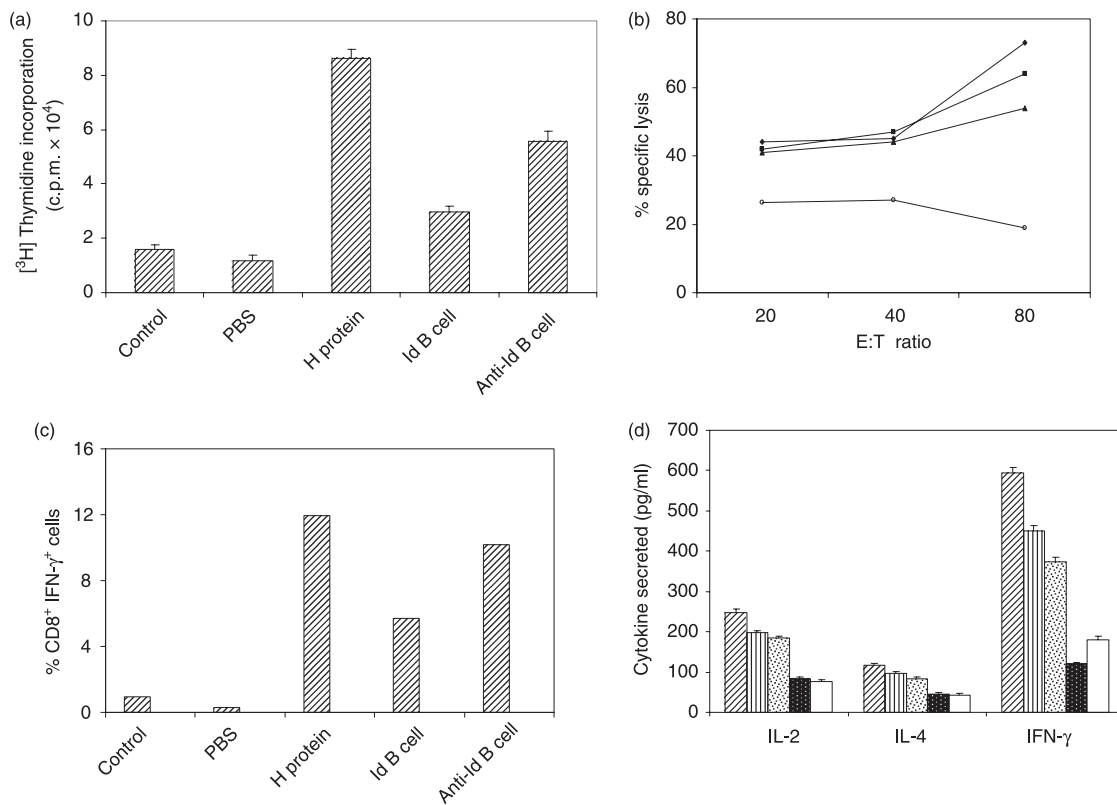
also secreted relatively higher amounts of IFN- $\gamma$  and IL-2, consistent with the proliferation assay results.

### Generation of B- and T-cell memory response after boosting of antigen-H-primed animals with anti-idiotypic B cells

To evaluate the role of anti-idiotypic B cells in the maintenance of B-cell and T-cell memory, mice were immun-

ized with H protein in Freund's complete adjuvant and, after 72 days, were boosted with H protein or irradiated H-specific idiotypic B cells or anti-idiotypic B cells. The memory T-cell recall response was monitored after 1 week of booster, using the splenocytes of these mice and *in vitro* proliferation assay, cytotoxic assay and cytokine response. The results showed that splenocytes from anti-idiotypic B-cell-boosted mice exhibited a secondary immune response similar to those mice that were boosted with antigen (Fig. 6a), whereas the cells from animals that did not receive booster immunization showed a smaller antigen-specific proliferative response. CD8<sup>+</sup> T cells from the lymph node cells of boosted mice, *in vitro* enriched in the presence of P815-H cells and IL-2, when used as effector cells, recognized the antigen-presenting cells and

exhibited killing activity. Cells from H-boosted, anti-idiotype-boosted mice showed the same levels of cytotoxicity followed by cells from idio-type-boosted mice (Fig. 6b). CD8<sup>+</sup> T cells from the lymph node cells of boosted mice secreted IFN- $\gamma$  after *in vitro* restimulation with P815-H cells, as shown by the intracellular cytokine staining (Fig. 6c). On restimulation with H protein, the splenocytes secreted high levels of IFN- $\gamma$  and considerable amount of IL-2, whereas IL-4 was not detected in significant amounts (Fig. 6d). The memory B-cell recall response was monitored by checking the presence of H-specific antibodies in the serum from immune animals. Serum from mice boosted with anti-idiotypic cells showed high titres of H-specific antibody, which was more than that in antigen-boosted mice (Table 2). Cumulatively,



**Figure 6.** Generation of memory T-cell response by anti-idiotypic B cells. Mice were immunized with H protein (50  $\mu$ g/mouse). After 72 days, a booster dose was given with H protein, irradiated H-specific idiotypic B cells (Id;  $2 \times 10^7$ ), anti-idiotypic B cells (anti-Id;  $2 \times 10^7$ ), normal B cells ( $2 \times 10^7$ ) or only PBS in Freund's incomplete adjuvant. After 1 week (a) *in vitro* proliferation of splenocytes was performed in the presence of H protein (50  $\mu$ g/ml). Data shown are the mean counts/min of triplicate wells. The result shown is representative of three independent experiments (b) Draining lymph nodes were collected from the above mentioned immune mice 1 week after booster. Lymph node cells ( $4 \times 10^6$  cells/well) (boosted with antigen,  $\blacklozenge$ ; anti-Id,  $\blacksquare$ ; Id,  $\blacktriangle$ ; normal cells,  $\circ$ ) were restimulated *in vitro* with irradiated P815-H cells and IL-2 (100 units/ml) as mentioned in the Materials and methods. A CTL assay was performed using these as effector cells and P815-H cells as targets. (c) Lymph node cells ( $4 \times 10^6$  cells/well) were restimulated *in vitro* with P815 cells expressing H protein or P815 cells ( $5 \times 10^6$  cells/well) transfected with vector alone for 8 hr in complete medium. Brefeldin-A was added in the last 6 hr. Surface staining for CD8 and intracellular cytokine staining for IFN- $\gamma$  were performed and analysed by fluorescence-activated cell sorting. The results shown are the percentages of CD8 and IFN- $\gamma$  double-positive cells. (d) From the *in vitro* proliferated splenocyte cultures (crossed bar, H; striped bar, anti-Id antibody; dotted bar, Id antibody; closed dotted bar, normal cells; open bar, PBS-boosted) cell-free supernatants were collected and were assayed for IL-2, IL-4 and IFN- $\gamma$ . Data shown are means of triplicate wells and the amount of cytokine (pg/ml) was calculated using cytokine standards.

**Table 2.** Generation of memory B-cell response by anti-idiotypic B cells as detected by measuring the amount of secreted antibody<sup>1</sup>

Antigen used for booster immunization	Antibody titre ( $\times 10^3$ ) <sup>2</sup>
H protein	> 16
Anti-idiotypic B cells	> 64
Idiotypic B cells	32
Irrelevant B cells	< 2
None	< 2

<sup>1</sup>Dilutions of sera collected from mice (as mentioned in legend of Fig. 6) were reacted with antigen (200  $\mu$ g/well of H protein).

<sup>2</sup>Data shown as titre are from the average optical density of triplicate wells normalized with readings for pre-immune sera.

boosting of antigen-immunized mice with anti-idiotypic B cells produced a secondary response comparable to that upon antigen boosting. Boosting with normal B cells did not result in a secondary response in animals demonstrating the specificity of recall response.

## Discussion

Anti-idiotypic antibodies with homologous sequences to specific regions of the immunogenic proteins of infectious organisms have been shown to functionally mimic these epitopes and to induce protective immune responses;<sup>21,22</sup> they have been used in experimental systems as surrogate vaccines against several bacterial,<sup>23</sup> viral<sup>22,24–27</sup> and parasitic organisms<sup>21,28</sup> and as several tumour-associated antigens.<sup>13,29</sup> It has been shown that a murine Ab<sub>2</sub> mimicking a peptide of hepatitis B surface antigen expressed epitopes for both B and T cells. The Ab<sub>2</sub> appeared to activate T cells through the same mechanisms as used by the nominal antigen.<sup>30</sup> Moreover, the Ab<sub>2</sub> elicited cellular and humoral responses in an out-bred strain of mice and also in a strain that was not responsive to the nominal antigen. Thus, the authors have postulated that Ab<sub>2</sub> vaccines containing specific epitopes capable of stimulating T and B cells might well induce a specific cellular and humoral immunity against the nominal antigen in a genetically diverse human population that has never been confronted with the antigen.

Operation of the idiotype network has been postulated in the control of autoimmune disease in animal models and patients.<sup>31–35</sup> Several studies<sup>36,37</sup> have shown that idiotypic networks have important roles in the control of a host's response to an antigen: regulating, enhancing or suppressing feedback mechanisms. Monoclonal anti-idiotypic antibodies fused to granulocyte-macrophage colony-stimulating factor have been shown to be capable of breaking tolerance to carcinoembryonic antigen in carcinoembryonic antigen transgenic mice.<sup>12</sup> Antigen-specific protective T-cell and B-cell immunity by an amplifying

anti-idiotypic response in multiple myeloma patients has also been demonstrated.<sup>38</sup>

The role of B cells in the presentation of idiopeptide to T cells has been shown in a transgenic mouse model where B cells present self-peptides in the absence of conventional antigen.<sup>39</sup> For the generation and regulation of specific immunity by anti-idiotypic and anti-idiotypic-derived peptidomimics, we postulate four possible mutually non-exclusive pathways.

- (1) Antigen-specific idiotype B cells, as well as their complementary anti-idiotypic B cells (carrying immunoglobulins on their surface that are specifically reactive against the idiotype determinant on the antigen-specific B cells) can undergo terminal differentiation into plasma cells upon recognition and contact with the idiotype determinants of the B-cell membrane-bound immunoglobulin.<sup>40</sup> Regulation of these cells is brought about by the engagement of their B-cell antigen receptor, leading to proliferation, differentiation and/or programmed cell death.<sup>41,42</sup>
- (2) Anti-idiotypic B cells can present peptide mimics of antigen to T helper and T cytotoxic cells thus priming them in the absence of antigen. Cytotoxic T cells complementary to the idiopeptide may recognize the cognate antigen or the idiopeptides derived from the antigen mimic or the anti-idiotypic immunoglobulin and kill the B cells presenting such idiopeptides.<sup>43–46</sup>
- (3) Receptor-mediated endocytosis followed by endosomal cleavage of the idiopeptide-anti-idiotypic complex can take place and peptides generated in the process can then be loaded onto MHC class II molecules and presented to CD4<sup>+</sup> T cells, switching on the T-cell and B-cell interactive idiotype cascade.
- (4) Apart from these, the idiopeptides can also be processed and presented *in vivo* by dendritic cells and by follicular dendritic cells in an MHC-restricted manner.<sup>6</sup>

To provide experimental evidence that supports the role played by the anti-idiotypic B cells in the generation of antigen-specific B-cell and T-cell memory responses and the regulation of B-cell homeostasis, it was imperative to study a single B-cell clone that was specific for a well-defined antigen. However, the isolation of such B-cell populations is difficult because of the presence of small numbers of idiotype and anti-idiotypic B cells, which limits their enrichment from the total cell population. The second alternative, which was ruled out, was the use of antigen-primed primary B-cell clones maintained *in vitro*, which cease to proliferate after a finite number of divisions in tissue culture. Use of Epstein-Barr-virus-transformed B-cell cultures was avoided because such cell populations are expected to present viral antigens that shift the experimental set-up away from natural conditions. We have therefore, used anti-idiotypic B-cell hybridoma as the surrogate B cells in this study.



In the present work, we have shown that on adoptive transfer of anti-idiotypic B cells carrying peptidomimics, antigen-specific B-cell and T-cell memory can be perpetuated. We have shown that anti-anti-idiotypic CD4<sup>+</sup> and CD8<sup>+</sup> T cells are generated in the body after the transfer of monoclonal anti-idiotypic B cells in syngeneic mice. We observed that when Ab<sub>1</sub> was introduced as a restimulant, significant amounts of cell proliferation occurred. This could be the result either of the anti-idiotypic cells used for primary immunization persisting in the body at the time of detection of anti-anti-idiotypic T and B cells, or of the generation of anti-anti-anti-idiotypic cells, which is the next stage in the cascade. The results show that idiotypic and anti-idiotypic T and B cells can coexist in the body and may form a dynamic system as a B-cell and T-cell idiotypic network. The anti-idiotypic-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells did proliferate relatively less efficiently against the synthetic peptides derived from anti-idiotypic antibody sequences, which is expected because the fragments of anti-idiotypic antibody may contain many fewer processed and presented epitopes compared to H protein or full-length anti-idiotypic antibody.

Furthermore, boosting with anti-idiotypic B cells generated antigen-specific memory T-cell and B-cell immune responses. This could be operative through direct B-cell–B-cell interactions or through presentation of idiopeptides by phagocytic antigen-presenting cells after phagocytosis of idiotypic T and B cells. The anti-idiotypic B and T cells generated after immunization with antigen are directed against antigen-reactive T cells, implying that the presence of an idiotypic B-cell–anti-idiotypic B-cell cascade is natural to the system after infection or vaccination.

Significant levels of IFN- $\gamma$  were secreted by Ab<sub>2</sub>-primed T cells upon *in vitro* restimulation with H, H-derived peptides or Ab<sub>2</sub>, although IL-4 was not detected, suggesting that CD4<sup>+</sup> cells induced by H were probably T helper type 1. Traditionally CD4<sup>+</sup> T cells function as helper cells for antibody production. Therefore D9D8 immunization/vaccination may prime T helper type 1 cells, which in turn may induce cytotoxic T-cell proliferation by secretion of cytokines such as IFN- $\gamma$ .

T-cell epitopes derived from the naturally processed self-peptides are the peptides most abundantly presented by MHC,<sup>47,48</sup> more than the peptides from pathogens. The anti-idiotypic antibodies, being self-generated, thus have more chance to act as elicitors of effective antigen-specific T-cell responses. The observed cytotoxic and lymphoproliferative responses indicate that anti-idiotypic B cells (Jerne cells) stimulate both T helper and T cytotoxic cells by virtue of their ability to present recycled or regurgitated peptidomimics of antigen to T helper cells through class II MHC and *de novo* synthesized peptidomimics of antigens to CTLs through class I MHC. Thus, the T-cell memory response can be perpetuated by anti-idiotypic Jerne B cells and these findings lend support to

the earlier proposed relay hypothesis for the perpetuation of immunological memory.<sup>2</sup> The results provide a mechanism for the maintenance of both idiotypic and anti-idiotypic B cells, which are generated in a cyclic fashion as has been proposed earlier.<sup>2</sup> This also means that B-cell and T-cell memory of longer duration could arise as the idiotypic cascade is triggered. Based on the structural information and on the recent study of altered peptide ligands,<sup>49,50</sup> we propose that peptidomimics in the CDRs of anti-idiotypic B cells, which may not be completely homologous to the original antigen peptide but which carry the structural complementarities,<sup>51</sup> will trigger idiotypic antigen-specific T cells.

In summary, the antibody repertoire contains the antigenic universe with an innate ability to react with the B and T cells of the self. The anti-idiotypic antibody functions as a surrogate antigen and idiotypic selection ensures that relevant B-cell clones are always maintained. The peptidomimics derived as a result of the degradation of anti-idiotypic antibody bring about idiotypic selection and activation of cognate CD4<sup>+</sup> and CD8<sup>+</sup> T cells, so an elevated population of activated T cells, the memory T cells, is always maintained. Thus the idiotypic selection of B cells and T cells containing the already randomly rearranged and mutated antibody and T-cell receptor genes ensures a specific memory response. The duration of the memory is dependent on the strength of the interaction of idiotypic and anti-idiotypic B-cell receptors and on the effective presentation of idiopeptides (peptidomimics) to cognate T cells.

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