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Idiotype and anti-idiotype specific T cell responses on transplantation with hybridomas reactive to viral hemagglutinin and human tumor antigen

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Abstract

B cell hybridomas expressing class I and II MHC molecules and producing antibodies directed against hemagglutinin protein of Rinderpest virus and human Mucin-1 have been used as surrogate B cells to study T cell responses against the antigens. The observed CTL and lymphoproliferative response indicates that anti-idiotypic B cells termed Jerne cells stimulate both T helper and T cytotoxic cells by virtue of their ability to present recycled or regurgitated peptido-mimics of antigen to T helper cells through class II MHC and de novo synthesized peptido-mimics of antigens to CTLs. Thus, 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 perpetuation of immunological memory (IM). © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Idiotype; Immunological memory; T cell responses; Relay hypothesis

1. Introduction

The nature of the immune system enables it to accommodate not only lymphocytes against an extremely large number of antigens but also requires that sufficient numbers of them with necessary specificities are present at any given time within the constraint of space. Following primary antigenic exposure, there is evidence for two discrete developmental pathways for antigen specific B cells [1], one which differentiates to the short living plasma cells and the other to the affinity-mature memory B cells [2]. The mechanisms that might be controlling the vast numbers of the memory pool against a vast array of antigens being committed to such an indefinite resting state is not clear. The immune system at any time is producing antibodies to match innumerable numbers of antigens causing massive proliferative drives in the effector pool. Besides these, there are those millions 'in waiting' naive cells being churned out from the bone marrow every day for the replenishment of the complete set of B cells that the immune system needs to possess. Antigen specific B cells as well as their complementary anti-idiotypic B cells (carrying immunoglobulins on their surface which are specifically reactive against the idiotypic determinant on the antigen specific B cells) can undergo terminal differentiation into plasma cells upon recognition and contact with the idiotypic determinants of the B cell membrane bound immunoglobulin [3]. Regulation is brought about by the engagement of B cell antigen receptor (BCR) and the progression of several signals for proliferation, differentiation or programmed cell death [4,5]. In addition, cytotoxic T cells complementary to the

Abbreviations: Id, idiotypic; CTL, cytotoxic T lymphocyte; RPV, Rinderpest virus; Mucin-1, human mucin-1 peptide; FDC, follicular dendritic cell.

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idiopeptide may recognize the cognate antigen or idiopeptide derived from antigen mimic or the anti-idiotypic immunoglobulin and kill the B cells presenting such idiopeptides [6–9]. Other investigations have shown that during natural progression of a B cell hybridoma as a tumor in a syngenic mouse system [10], the B cell idiopeptides function by recognizing and interacting with antigenic epitopes. Receptor mediated endocytosis and endosomal breakdown of idiotype–antigen complexes into peptides takes place, which are then loaded onto class II MHC and presented to $CD4^+$ T cells [11]. Since in an individual syngenic system, an idiopeptide is an endogenous 'apparently non-self' product of a B cell, it is likely to have interactions with MHC I molecules during its passage through endoplasmic reticulum and Golgi complex [12]. Idiopeptide has also been used as a marker peptide of the processed immunoglobulin, since it can be tracked down and used as the model self protein to study the induction of auto-reactive T cells [13]. Recently the role of B cells in idiotype presentation to T cell was reported using transgenic mouse approaches [14] where B cells presented self-peptides in the absence of conventional antigen.

There is evidence that interactions between B and T cells are important in memory generation and maintenance [15]. While T cell memory has been documented in the absence of B cells [16] these animals are not T cell deficient and it is envisaged that presentation of persisting antigen or idiopeptides may be taken over in these models by professional antigen presenting cells such as macrophages and follicular dendritic cells. There is evidence now that $CD4^+$ and $CD8^+$ T cell memory can be propagated in mice deficient for MHC class II and I molecules [17,18]. However, these experimental systems do not rule out the possibilities of B–T cell interactions through class I molecules in class II deficient mice and through class II pathways in the MHC I deficient mice. Recently a hypothesis termed 'Relay hypothesis' has been proposed to explain the perpetuation of immunological memory (IM) [19]. According to this hypothesis, IM is driven by two types of complementary B cells; one which is antigen specific and the other, idiotype specific (or producing anti-idiotypic antibodies). The antigen specific memory cell has been named as Burnet cell whereas anti-idiotypic cell with specificity for idiotype on Burnet cell is named as the Jerne cell. It has been proposed that due to the interaction of Burnet and Jerne cells, both cells proliferate and thus initiate a cascade where IM is perpetuated which does not require long living memory cells or persistent antigen. In order to provide experimental evidence supportive of the role played by the membrane bound and the secreted idiotypic peptides of the B cells in the generation of antigen specific T cell memory responses in the absence of antigenic restimulation and the regulation of B cell homeostasis, it was imperative to study a single B cell clone specific for a well defined antigen. However, the isolation of such B cells populations is difficult due to the very low numbers of idiotypic and anti-idiotypic B cells which may be present thereby limiting the enrichment of such B cells by Flow Cytometry aided sorting. The second alternative which was ruled out is the use of antigen primed in vitro maintained primary B cell clones which cease to proliferate after a finite number of divisions in tissue culture. Use of Epstein Barr virus transformed B cell cultures was avoided since such a cell population is expected to present viral antigens which shifts the experimental setup away from the natural conditions. We have therefore, used two B cell hybridomas as surrogate B cells in this study. One of the postulates of the hypothesis [19] is that the presentation of idiopeptides of B cells can perpetuate T cell memory by activating specific T helper cells. The cytotoxic T cell memory can also be perpetuated due to the activation of CTL by the peptido-mimics of antigen by anti-idiotypic B cell (Jerne cell). The ability of B cells to process and present antigens is well studied [20]. We have recently shown that immunization of syngenic mice with cell preparations of antigen, idiotypic antibody or anti-anti-idiotypic antibody generate idiotype and antigen specific T cells [21]. This work attempts to test if antigen specific T cell responses are generated when B cells present idiotypic determinants to T cells and the idiotypic cascade is switched on. These observations suggest that antigen specific CTL and proliferative response are generated when hybridoma cells generated against the antigen is used as surrogate B cells by transplanting syngenic BALB/c mice with the hybridoma.

2. Materials and methods

2.1. Hybridoma and cells

A12A9, a B cell hybridoma line of BALB/c origin was generated against the purified recombinant hemagglutinin (H) protein of Rinderpest virus (RPV) [22] using SP2/0 as the myeloma fusion partner. The antibody isotype was IgM,κ. E2A3 is also BALB/c derived hybridoma, secreting IgM antibodies against a synthetic 60 mer peptide of human Mucin-1, containing three repeats of the following 20 amino acid sequence (VTSAPDTRPQAPGSTAPPAHG) and was generated using the same fusion partner. Both the hybridoma cell lines were maintained in IMDM supplemented with 10% heat inactivated fetal bovine serum (FBS), 2 mM glutamine, 10 μg/ml gentamycin sulfate for over 2 years without any apparent phenotypic changes.

P815 ($H\text{-}2^d$) murine mastocytoma cells were cultured in RPMI- 1640 (Gibco BRL, USA) supplemented with 5% heat inactivated FBS (Life Technologies, USA).

2.2. Mice and immunizations

Al2A9 or E2A3 cells harvested 12–16 h after seeding in fresh medium, were resuspended in PBS and injected subcutaneously into 8-week-old female BALB/c mice (obtained from the Central Animal facility, Indian Institute of Science) at a dose of 1×10^6 cells per animal for tumor formation. Sets of two mice per experiment were used. The animals were usually euthanised 20 days post transplantation by cervical dislocation and the spleens collected for in vitro T cell assays. Experiments were repeated thrice for the analysis of statistical significance.

2.3. Purification of T cells

T cells were purified as described earlier [23]. Briefly, single cell suspensions of splenocytes were subjected to lysis of RBC by ACK lysis buffer (150 mM NH_4Cl, 1 mM $KHCO_3$ and 0.1 mM Na_2EDTA) and plated on FBS coated petriplates (100 mm tissue culture plates were coated with 2 ml of FBS and kept for binding at 37 °C for 1 h) to remove adherent macrophages. The non-adherent cells were subjected to Ficoll-Hypaque gradient centrifugation at 3000 rpm for 20 min at RT. The buffy coat was washed and plated on protein A coated petriplates (prepared by coating each 100 mm pertridish with 100 μg of protein A dissolved in serum free IMDM) for B cell adherence at 37 °C for 1h. The non-adherent cells were removed, washed with medium and loaded on to a pre-calibrated nylon wool column. The non-adherent T cells were collected with repeated washes with medium.

2.4. Preparation of stimulator cells and CTL targets

Stimulators and targets for the anti-idiotypic assays were hybridoma cells harvested in log phase of growth and washed with serum free medium. The cells were irradiated at 4500 rads for 3 min to arrest DNA replication. The antigen specific proliferation and CTL assays require the use of replication-arrested antigen expressing cells. For the RPV H assays we used the full length RPV H gene (pRBH3.41 clone is a kind gift of Dr T. Barrett, Institute for Animal Health, Pirbright, UK) cloned in an eukaryotic expression vector pCMX [24] to transfect the P815 cells using Lipofectamine reagent. The cells were irradiated 12 h post transfection at 3500–4000 rads for 3 min and used for assays 24 h post-infection. For the Mucin-1 specific assays the cells in serum free medium were pulsed with the peptide (kind gift of Dr Dick Schol, Vrije University Hospital, Amsterdam) at a concentration of 10^{-7} M in 35 mm dishes at 37 °C in a CO_2 incubator. The cells were irradiated 12 h post pulsing as described before. The assays were setup 12–16 h after the incubation with the peptide.

2.5. T lymphocyte proliferation assays

A12A9 or E2A3 hybridoma cells or P815 cells transfected with the full length RPV H or Mucin-1 peptide pulsed P815 cells were gamma irradiated at 3500 rads for 3 min before use as stimulators. Stimulators were plated in 96-well flat bottom tissue culture plate in different numbers ranging from 2 to 8×10^3 cells/well. Responder T cells ($1 \times l0^5$) isolated as described earlier were added to these stimulators in a final volume of 200 μl and incubated in a 37 °C humidified CO_2 incubator for 5 days. Control assays were set up using heterogeneous hybridoma or untransfected P815 cells as stimulators. Tritiated thymidine (0.5 μCi/well, specific activity 6500 mCi/mmol, Bhabha Atomic Research Center, India) was added 16 h before harvesting the cultures. The incorporated radioactivity in triplicate wells was measured in a scintillation spectrometer. Experiments were preformed at least three times with triplicates for all samples. Stimulation Index was calculated by:

$$\mathrm{SI} = \frac{\text{mean cpm of antigen stimulated wells}}{\text{mean cpm of control wells}}. \quad (1)$$

2.6. CTL assays

Three weeks after the immunization with the hybridoma cells splenic lymphocytes were harvested as described above and restimulated in vitro with varying numbers of hybridoma cells, antigen expressing cells or peptide pulsed cells. After 5 days CTL activity was assessed in the stimulated cells using A12A9, E2A3, P815 cells transfected with pCMX plasmid containing the RPV H gene or P815 cells pulsed with Mucin-1 peptide at different effector:target ratios. CTL activity was measured by a CTL detection kit (Boehringer Mannheim) as described in manufacturer instructions. Briefly, the effector and target cells were plated in triplicates in different effector:target ratios in 96 well dishes for 10–12 h. Hundred microliter of the cell free culture supernatants were collected in 96 well plates and l00 μl of the dye substrate was added to each well. The extent of lysis of the targets was proportional to the LDH released from the cells and was detected in the culture supernatants using a color reaction read at 600 nm. Experiments were preformed at least three times with triplicates for all samples. The percent specific lysis was calculated by the formula

$$\%\ \text{specific lysis} = \frac{(\text{experimental release} - \text{spontaneous release})}{(\text{total release} - \text{spontaneous release})} \times 100. \quad (2)$$

The MHC class I restriction of the CTL assay was demonstrated by incubating the CTL targets with 1:100

final dilution of anti-murine MHC I antibodies (kind gift from Dr Dipankar Nandi, Deptartment of Biochemistry, Indian Institute of Science) in the form of ascites fluid recognising the H-2^d determinant (HB79-ATCC).

3. Results

3.1. Generation of anti-Id T helper and T cytotoxic cells in response to live hybridoma immunization

Assigning a function to the processing and presentation of the idiopeptides by the B cells to their cognate T cells was possible only on the assumption that the idiopeptides within the B cell were being recognized as 'foreign' by T cells. The MHC II associated idiopeptides derived from the membrane bound IgM or the internalized IgM are processed by the endosomal compartments and displayed in context with MHC II on the surface of the hybridomas. The ability of hybridoma cells as surrogate B cells to function as antigen presenting cells was evaluated by staining with either anti-murine H-2^d or anti-murine I-A/I-E antibody followed by confocal microscopy. More than 90% of the cells were found to express the MHC molecules on the cell surface making these hybridoma cell lines suitable as surrogate B cells to present idiotypic determinants both by MHC I and II antigen presentation pathways (data not shown). When mice were injected with such idiopeptide bearing hybridoma cells growing as solid tumors, the animals produced T cells which proliferated in vitro upon restimulation with the homologous, irradiated hybridoma cells (Fig. 1(a)) but not with non-specific hybridoma cells. The proliferation or the stimulation index indicated that T cells recognizing the idiopeptides on each of the hybridomas were indeed generated, and therefore, a CTL assay was performed to turther characterize the nature of these activated T cells. As shown in (Fig. 1(b)) A12A9 hybridoma immune T cells effectively killed A12A9 targets but not E2A3 targets in a MHC I restricted fashion since lysis was abrogated by the pre-treatment of the target cells with anti-MHC I antibodies.

3.2. Demonstration of anti-anti-Id or antigen specific T cells

In order to determine if the maintenance of the B cell homeostasis is also T cell dependent, T cell functions were studied. In these assays, idiopeptide immune T cells proliferated in response to cells expressing antigenic peptides or antigen mimics equally well and were proficient in killing antigen expressing cells although the immune animals had never been exposed to the actual antigen. As can been seen from the specific thymidine uptake (Fig. 2(a)) as well as class I restricted CTL generation (Fig. 2(b)), the animals showed sub-

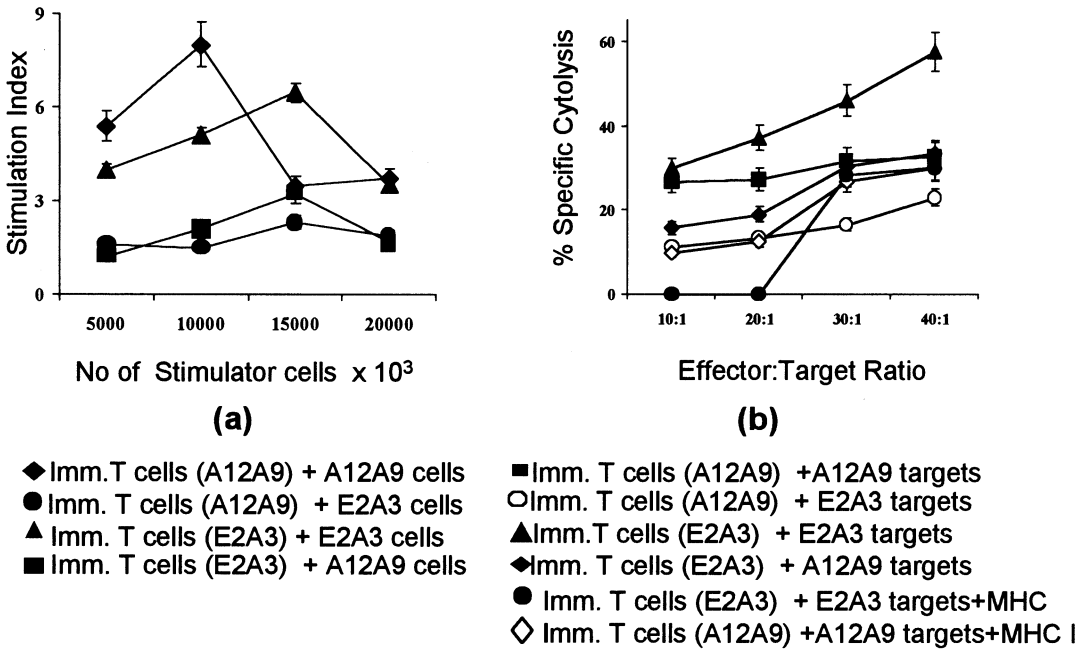

Fig. 1. Detection of idiotype specific T lymphocytes in syngenic mice on A12A9 or E2A3 cell transplantation. (a) Immune splenic T cells were in vitro re-stimulated with 5×10^3, 10×10^3, 15×10^3 and 20×10^3 homologous or heterologous hybridoma cells and their proliferation was measured in terms of radioactive thymidine incorporation. (b) In vitro re-stimulated lymphocytes were used to measure their ability to lyse specific idiopeptide bearing targets in a MHC restricted manner in a LDH release CTL assay.

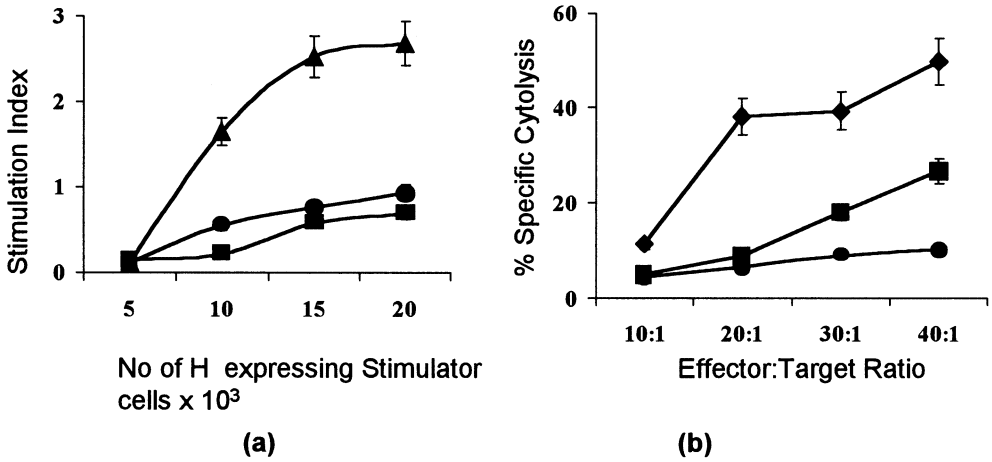

Fig. 2. Detection of anti-Id or antigen specific T lymphocytes in syngenic mice upon A12A9 cell transplantation. (a) Represents the proliferation profile in A12A9 hybridoma cell immune T cells upon in vitro stimulation with the 5×10^3, 10×10^3, 15×10^3 and $20X10^3$ P815 cells expressing RPV H (▲) and untransfected (■) and vector transfected (●) stimulator cells serve as the non-specific stimulation control. (b) CTL assay showing the generation of RPV H (◆) specific CTL effectors in the absence of antigen immunization. The lytic activity is inhibited in the presence of (■) anti-MHC I antibodies. Vector transfected targets (●) served as background lysis controls.

stantial levels of T cells primed against the antigen by virtue of the selective proliferation of T cells by the antigen mimic present on the anti-Id B cells. T helper cells provide selective help to such B cells by their direct or bystander help in the case of anti-Id B cell. This result supports the 'Relay hypothesis', especially since it is a pointer not only to an additional mechanism by which the idiotypic network brings about immunoregulation but also indicates how antigen specific T cell might be propagated in the absence of the antigen or long living memory cells.

4. Discussion

The B cells have been well accepted as good antigen presenting cells in vivo [25,26]. The use of hybridoma as surrogate antigen presenting B cells enabled the design of experiments where extensive B and T cell functional assays requiring large-scale B cells secreting Ab1 were envisaged. Such requirements could not be met by either primary cultures, which do not replicate in tissue culture beyond a fixed number of passages or EBV transformed B cell clones, which are likely to express endogenous viral antigens. FACS sorted lymphoid B cells which may have been modified during staining procedures also pose a likely problem due to binding of the antibodies and inadvertent activation etc. The results described here provide evidence for the role of B cell bound immunoglobulin molecules and their idiotypic determinants in T–B cellular interactions that culminate in up or down regulation of an immune response. Although in vivo, such idiopeptides may also be processed and presented by dendritic cells and follicular dendritic cells [27,28], B cells which synthesize and metabolize such peptides are hypothesized to play a major role during the memory maintenance phase where the capture and processing of free antigen antibody complexes by the FDC are presumably at very low levels. It is evident from the results described here that an antigen specific IgM hybridoma, which evokes a primary or a secondary response in a syngenic mouse background, is the idiotype processing and idiopeptide presenting center and therefore, is a good system to study epitope and clonal populations of B and T cell responses. We have earlier observed the generation of the anti-Id antibodies as well as the anti-anti-Id antibodies and B cells carrying these antibodies [29]. Since the idiotypic region is recognized as a non-self-determinant by the B cell arm of the immune system, we expected the T cell responses to be similarly elicited by B cells acting as the antigen presenting cells where they can present the idiopeptide, both on MHC I and MHC II molecules [21,29,30]. This study describes the Id as well as the anti-Id specific T cell responses generated in response to the hybridoma secreting Ab1 (idiotypic antibody). It was found that these responses are MHC restricted and provides a mechanism to explain how the generation of such self-reactive T cells may play a critical role in the regulation of the B cell population.

It is evident that the idiopeptides on both A12A9 and E2A3 are involved in interactions with regulatory $CD4^+$ and $CD8^-$ and that it is derived from de novo synthesized and processed forms of the IgM. Although recognition of the idiopeptide by the Id specific T cells has been shown by others [31–33], this is the first report where the simultaneous generation of the anti-Id or the antigen specific T cells has been demonstrated in

B cell immune syngenic mice in the absence of antigen immunization.

The experiments reported in these studies use two hybridoma directed against two different antigens (A12A9 reacts against Rinderpest virus hemagglutinin and E2A3 reacts against a 20 mer synthetic peptide of Mucin-1). Both these hybridoma express class I and MHC on their surface and are expected to present endogenous and exogenous peptides to T cells. The results clearly show that the hybridoma cells present their idiopeptides to T helper and T cytotoxic cells. This is possible because the idiopeptides of the antibodies generated by the hybridoma are recognized as 'apparently non-self' and therefore, are capable of mounting a T cell response in a MHC restricted manner. However, the question remains as to the origin of T cell responses against the cells transfected with antigen or pulsed with the antigenic peptide. These mice were never exposed to these antigens and therefore, have not been primed by the antigens themselves. A model depicting the proposed mechanism for the generation of antigen specific T cell responses is shown in Fig. 3. It appears that the antigen specific response can only be produced if antigen mimics are generated in the anti-idiotypic B cells which recognize the hybridoma, receive both specific and bystander T cell help, proliferate and synthesize anti-idiotypic antibody. We have seen the synthesis of anti-idiotypic antibodies in BALB/c mice transplanted with hybridoma or antibodies [30]. Therefore, it stands to reason that the anti-idiotypic B cells which have been termed as Jerne cells present peptido-mimics of antigen to T helper and T cytotoxic cells thus priming them in the absence of antigen. Thus it provides an interesting mechanism for maintenance of T cell memory which is primarily B cell driven. This work provides experimental support for the relay hypothesis proposed for perpetuation of IM [19].

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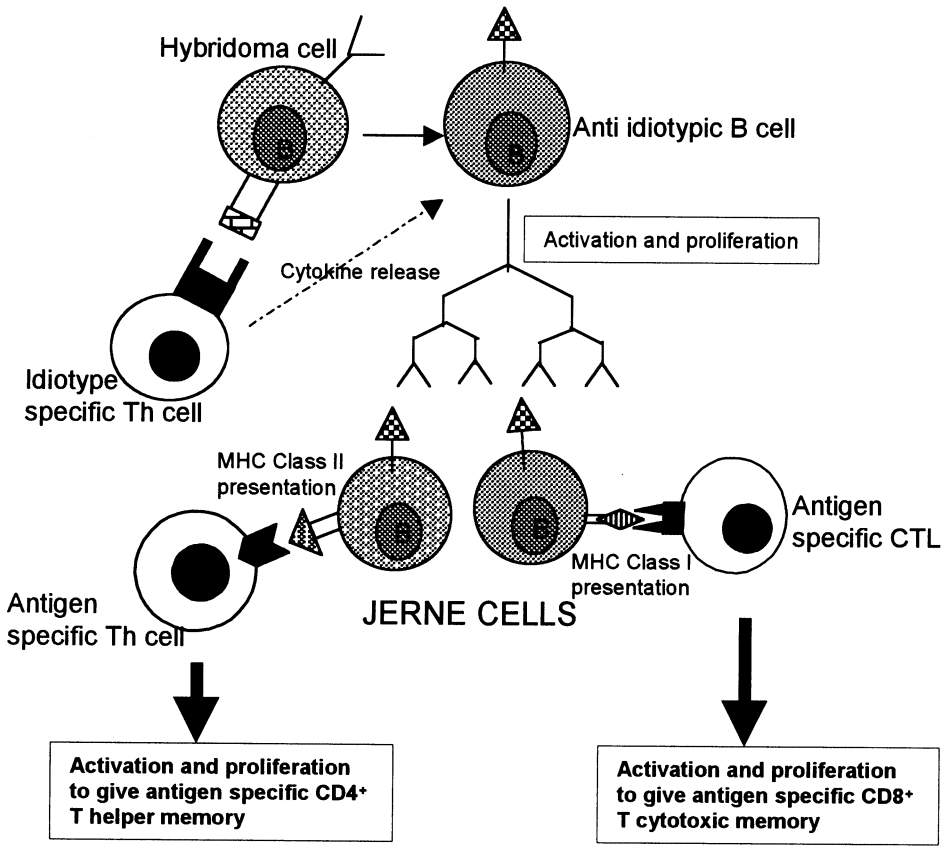

Fig. 3. Proposed model for the generation of antigen specific T cell responses on hybridoma transplantation.

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