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Supporting Biomedical Investigation

Research Grant Application

CURRENT DATE: November 8, 1994

TITLE OF STUDY (*DO NOT EXCEED 70 CHARACTERS*)
Mice with an inducible monoclonal B cell compartment

KEY WORDS (LIMIT TO FIVE)
immunological memory / autoimmune disease / somatic mutation / inducible expression

PROPOSED START DATE	PROPOSED DURATION (YRS) 1, 2 OR 3	FUNDS REQUESTED (INCLUDING INDIRECT COSTS)	YEAR 1	YEAR 2	YEAR 3
7/1/95	3 yrs.	TOTAL REQUEST (page 5):	$103,154	$107,740	$113,898
		PERM. EQUIPMENT (page 5):	None	None	-0-

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PRINCIPAL INVESTIGATOR. I have read the Council's Statement of Policy and agree to its terms and conditions. Also, I accept responsibility for the scientific conduct of this project and will provide progress reports when requested. I will acknowledge support by the COUNCIL FOR TOBACCO RESEARCH in publications resulting from this work.

M Wabl Nov 7,94

Signature Date

RESPONSIBLE INSTITUTIONAL OFFICIAL. I have read the Council's Statement of Policy and agree to its terms and conditions regarding any award made to the Principal Investigator.

Peter S. Nishi NOV 2 9 1994

Signature Date

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1. a. P.I. : Prof. Matthias Wabl
   b. Institution : University of California, San Francisco
   c. Title of Project : Mice with an inducible monoclonal B cell compartment

d. Clearly summarize BACKGROUND, WORKING HYPOTHESIS and BROAD GOALS of the project. Do not use additional pages.

Ig genes. Immunoglobulins (Ig's), consist of heavy (H) and light (L) chains, on each of which variable (V) and constant (C) regions can be distinguished. In the germ line, there are no functional genes encoding the V regions of Ig chains, only gene segments – V_H, D, and J_H at the H chain locus, and V_λ and J_λ or V_κ and J_κ at the two L chain loci. In pre-B cells, these are joined to enable a small B cell to produce the H and L chains of its antibody [Tonegawa 1983]. Allelic and isotypic exclusion reduce the number of functional genes to one encoding an H chain and one encoding an L chain, thereby ensuring monospecificity of B cells [Pernis *et al.* 1965; Weiler 1965].

Memory. Immunological memory was, historically, the first feature of the immune system to be recognized. Now one must distinguish between B cell memory and T cell memory. It is generally, though not universally, accepted that there exist long-lived (up to the lifetime of the animal) memory T cells that do not need antigen for their long-term survival. The situation with regard to B cell memory is also not entirely clear. Just as with T cells, it is thought that primary encounter with antigen stimulates specific B cells not only to differentiate into plasma cells, but also to give rise to populations of memory cells that differ from virgin B cells in many characteristics including their life span and the fact that they have accumulated somatic mutations in the V exons. When re-exposed to antigen, memory cells generate secondary responses, which consist mostly of the IgG subclasses and which are increased in rate, titer, and affinity.

Lineage. A current view of memory cell generation is that during the expansion of antigen-stimulated B cells some cells are set aside as memory cells, whereas others differentiate into plasma cells. This could be achieved by asymmetrical division or by differential influence of outside factors, such as lymphokines and T helper cells. However, it has also been proposed that memory cells arise from a lineage different from the cells that constitute the primary response [Linton et al. 1989].

Ig isotypes as markers. The isotype of an Ig molecule is determined by C_H. Naive B cells, which have never seen antigen, express IgM (and usually IgD as well). After antigenic stimulation, some B cells switch to other isotypes, principally IgG. IgG-bearing cells seem to be mostly memory cells, but the converse is not necessarily true, even though early experiments seemed to point that way [Coffman & Cohn 1977]. It has been argued that the 2% of splenic IgG^+ cells are too few to store memory for the many antigens a mouse has encountered; furthermore, this number should increase with age of the mouse, which is not the case [Gray 1993]. To date experiments on isotypes as memory cell markers are contradictory. In one report, upon adoptive transfer, only IgD^+ cells, but not IgM^+ or IgG^+ could propagate memory [Zan-Bar et al. 1979]. In another report, IgD^+ cells could transfer memory, but were not self-renewing; they gave rise to IgD^- cells that could propagate memory [Herzenberg et al. 1980; Black et al. 1980]. In any case, B cell memory and affinity maturation do not seem to be grossly disturbed in mice deficient for IgD expression [Nitschke et al. 1993; Roes and Rajewsky 1993].

Somatic mutations in the V exons. As mutated V exons are at present the only reliable marker of memory B cells [Gray 1993], *bona fide* memory cells are now almost defined as cells that carry somatic mutations in the V exons of the immunoglobulin genes [Kocks and Rajewsky 1989; McHeyzer-Williams et al. 1991]. The large number of mutations stems from a combination of (i) hypermutation in the V exons and (ii) cellular selection for better antigen binding.

Continual antigen dependence. The conventional view of memory cells is that once generated, they are maintained in the absence of antigen until they are re-stimulated to give rise to the secondary response. However, it has been proposed that the maintainance of memory B cells requires continuous stimulation by antigen [Gray & Skarvall 1988]. A monoclonal mouse with its H chain transgene under an inducible promoter would allows us to study this question from the antigen receptor side.

2. List the SPECIFIC AIMS of this research project (as opposed to the broad goals presented in Item 1). Do not exceed the space allotted below.

Our aims are: (i) to produce monospecific mice in which the Ig production can be turned on and off at will (the "memory replacement mouse"), (ii) to characterize these mice with regard to their B cells and the Ig they produce, and (iii) to use these mice for experiments on B cell memory – to determine whether antigen must be present continuously for memory to persist and to search for surface markers of memory B cells.

The very large diversity of antigen receptors that makes B cells so interesting also hampers their study in the intact mouse. We propose to use a new approach to study B lymphocyte memory. Using targeted homologous recombination, we will replace the stretch of genomic DNA containing all of the J_H gene segments with an assembled V_HDJ_H segment encoding the variable region of a particular immunoglobulin heavy chain. When paired with most any immunoglobulin λ light chain, this VDJ segment is specific for the hapten NP.

In order to mimic the situation in a normal B lymphocyte, where only one allele is active, this mouse will be crossed to the GenPharm "double knockout" mouse, in which both the H chain and the κ L chain loci have been rendered non-functional. The heterozygous F1 will be used for memory experiments. The use of the knockout mouse also ensures that essentially all B lymphocytes express an H chain V region derived from the inserted V_HDJ_H segment and a λ L chain.

Such an inducible monoclonal mouse would also be valuable for many other experiments in basic B cell biology, including studies of cell kinetics and turnover during B cell ontogeny. It could also prove to be valuable for studying the effect of various concentration of an anti-self antibody on the onset of an autoimmune disease, *i.e.*, lupus and rheumatoid arthritis. Even though the focus of this grant proposal is on memory experiments, we hope that these studies will establish the use of inducible monoclonal mice harboring any other specificity as models for diseases where a specific antibody response is suspected to be a cause.

3. SUPPORTING DATA, EXPERIMENTAL DESIGN and PROCEDURES. Do not attach more than six (6) additional pages (3a-3f). All figures, charts, tables and references must fit within pages 3 - 3f.

How do we assure monoclonality? The hapten NP (4-hydroxy-3-nitrophenyl) acetyl induces an immune response restricted in idiotype [Imanishi & Mäkelä 1974; Karjalainen 1980; White-Scharf & Imanishi-Kari 1981, 1982]. V_H segments encoding these idiotypes, including V_H17.2.25 [Loh *et al.* 1983], have been cloned. Antibodies produced during a primary response almost all contain λ. There are two V_λ segments in the mouse [Apella 1971; Schulenberg *et al.* 1971; Azuma *et al.* 1981; Blomberg *et al.* 1981; Miller *et al.* 1981; Selsing *et al.* 1982; Elliot *et al.* 1982; Reilly *et al.* 1984]; *Ca.* 80% of the λ chains contain V_λ1 (joined to C_λ1 or C_λ3) and 20% contain V_λ2. While 17.2.25 H chain produces anti-NP antibodies when it combines with a λ L chain, it has not yet been tested whether V_H17.2.25 associated with V_λ2 is also NP specific. Transgenic mouse lines carrying V_H17.2.25 were generated by Grosschedl *et al.* [1984]. The μ transgene encodes an H chain that, combined with λ1 L chain, is specific for NP [White-Scharf & Imanishi-Kari 1982; Loh *et al.* 1983]. When compared to sera of normal mice, non-immune sera of the transgenic mice had dramatically increased levels of NP-binding antibody containing λ L chains. The serum levels were up to 400-fold higher than those of normal mice.

It is an oversimplification to call these mice "monoclonal". A B cell clone is a product of a single productive rearrangement at the H chain locus and an L chain locus. Although the H chain gene is preformed in these mice, the λ locus must be rearranged. There are two V_λ gene segments. V_λ2, which differs from V_λ1 by one amino acid in a hypervariable region, might have another specificity. However, any given cell will express either V_λ1 or V_λ2; unlike conventional transgenics, these mice will have no B cells with more than one antibody. Other variations are introduced by the fact that there are three J_λ's. Finally, even if the same V_λ rearranges to the same J_λ, clones with independent rearrangement events will differ in junctional diversity and somatic point mutations. We do not anticipate a major drawback caused by these presumably minor variations, and indeed, as will be seen below, we will depend on somatic mutations at the heavy chain locus to distinguish a primary from a secondary response.

Concerning differences in regard to regular mice. Whenever one generates a transgenic mouse, one has to ask whether or not conclusions reached with the transgenic also apply to normal, non-transgenic, mice. There are two obvious differences between our mice and normal mice: absence of VDJ rearrangement and near monoclonality. But formation of memory B lymphocytes ought not to be influenced by the lack of VDJ rearrangement; when antigen triggers a small, resting B lymphocyte to form a clone and set apart some memory cells, the VDJ exon has been formed in both transgenic and normal mice. If network effects play a role in memory, our mice could behave very differently in a secondary immune response from regular non-transgenic mice. We think this is quite unlikely, but even if our experiment "fails" for this reason, it would still be worthwhile. Since we will test the mice under continued "induced" condition, i.e., with Ig continuously present, we will know whether or not memory follows the same temporal and qualitative pattern of a regular immune response.

Supporting Data

Validation of the inducible gene expression system

The tet-responsive promoter system. A recently described inducible promoter can be regulated in activity over a range of up to five orders of magnitude and works in a variety of human and murine cells. [Gossen & Bujard 1992]. It also works well in transgenic mice [Furth et al. 1994]. The control system consists of two elements (Figure 1). One element, a fusion protein consisting of the *tet* repressor of *E. coli* and the activating domain of virion protein 16 of herpes simplex virus (HSV), is constitutively expressed in mammalian cell lines and can also be expressed in transgenic mouse lines [Furth et al. 1994]; it constitutes a tetracycline-controlled transactivator (tTA). This transactivator stimulates transcription of the other element, a minimal promoter sequence derived from the human cytomegalovirus (CMV) promoter IE combined with *tet* operator sequences. These promoters are virtually silent in the presence of the tTA plus tetracycline, which prevents the tetracycline-controlled transactivator (tTA) from binding to *tet* operator sequences.

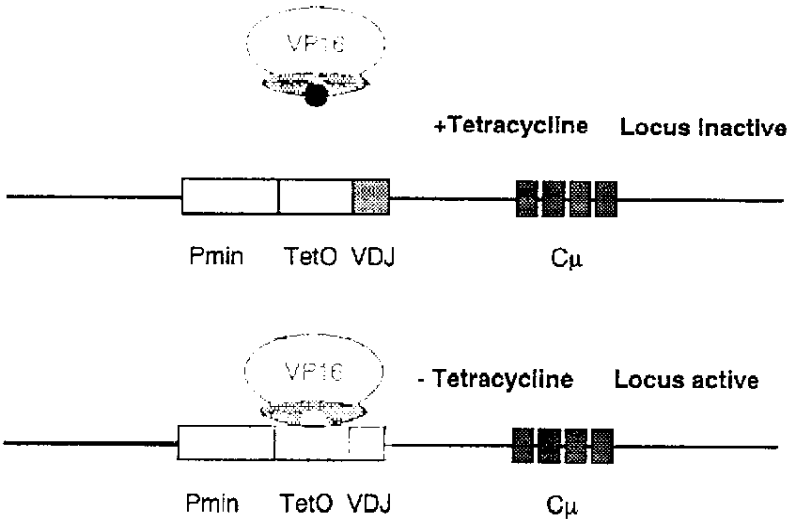

Figure 1. Schematic representation of a tetracycline-repressible expression system for generating a "memory mouse". A minimal promoter (Pmin) and elements of the bacterial tet operon are cloned to the immunoglobulin heavy chain coding sequences. In a cell line (or mouse) constitutively expressing HSV VP16 fusion protein, which functions as a transactivator (tA), heavy chain will be expressed so long as there is no tetracycline present in the medium (or drinking water). Tetracycline binds to the tA, and thus its presence represses heavy chain expression.

Inducible luciferase transgenic mice. We have generated mice transgenic for the transactivator and mice transgenic for the luciferase gene under the tet-responsive promoter.

Doubly transgenic mice were produced by mating the two strains. Depending on the mouse and tissue, the induction can be over four orders of magnitude; the expression of uninduced luciferase is often at background levels; *i.e.*, similar to that in non-transgenic mice with luciferase activity of <0.01 rlu/μg protein. In doubly transgenic mice, the levels are 0.2 rlu/μg to <0.02 rlu/μg before induction and increase to 1000 rlu/μg after induction. As these mice are not yet inbred, such variability is not too surprising. 1000 rlu/μg protein corresponds to *ca.* $2x10^4$ molecules/cell, and there are *ca.* 10^5 Ig molecules on the membrane of a B cell. Even though luciferase and immunoglobulin production cannot be compared in a straight fashion, the order of magnitude of the induced gene product is correct. In a non-induced cell, there may be fewer than 10 molecules luciferase.

When primary fibroblast cells were recovered from another mouse line transgenic for the luciferase gene, there was no luciferase activity, with or without tetracycline present in the culture medium. After transient transfection of the tTA construct luciferase expression increased by several orders of magnitude. This test can also be used to select the most promising breeder.

A technical improvement. Tetracycline can be administered via the drinking water, but another way has now been studied in detail. Using a trochar according to the manufacturer's directions, we implanted slow release (0.7 mg/day) tetracycline pellets (Innovative Research) subcutaneously in the shoulder. The concentration of tetracycline in serum was 0.5 to 3 μg/ml, well above that needed for indicator gene repression.

Transactivator driven from the Ig H chain promoter. A mouse transgenic for the tTa under the control of the Ig H chain promoter will be needed for the memory replacement mouse. A Bam HI - Dra II fragment , isolated from a pμ construct, contains all relevant promoter sequences, *i.e.*, Py, heptamer, octamer, TATA and transcription initiation; it was cloned into a bluescript KS- vector at the Eco RV site. From there, the Xho I - Eco RI fragment was cloned into the corresponding sites of pUHD 15.1. This construct was first tested in cell lines and then injected into oocytes to generate transgenic mice. When these mice were crossed to an indicator luciferase strain, luciferase activity was tighly regulated by tetracycline.

Generation of a non-inducible monoclonal mouse

When this project was conceived, the tetracycline-responsive inducible gene expression system was new and not validated for use in transgenic mice. We decided to develop a simple non-inducible system in which the J_H region was replaced by V_H17.2.25 under the control of its endogenous promoter. This was intended as an exercise to prove that we mastered the homologous replacement technology and to provide a backup system in case the inducible system was not suitable.

Replacement construct. The 16.3 Kb plasmid t-v.1 (Figure 2) contains a rearranged μ H chain gene; all exons encoding the secreted form are included but not those encoding the membrane exons. In addition, it contains the selectable markers *neor* and *tk*, both of which have the opposite orientation as the inserted anti-NP μ gene. The rearranged segment encoding the V region contains V_H17.2.25 joined to a D segment and to J_4. It is flanked by stretches of DNA homologous to those 5' and 3' of the stretch containing the J_H segments in the normal Ig H chain locus. The long 3' stretch contains the major intron and C_μ. The shorter 5' stretch contains DQ52, the most proximal D segment.

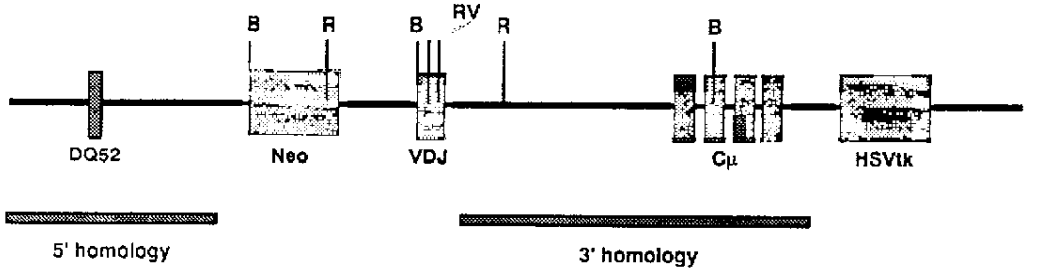

Figure 2. Targeting vector t-v.1. Restriction enzyme sites: B = BamH1; R = EcoR1; RV = EcoR5.

The 5' homologous region, a 1.6 Kb Hind3-BamH1 DNA fragment containing the DQ52 segment, was cloned into the same sites of Pmc1Neo (Stratagene). Subsequently, the Xho1 site 5' of DQ52 segment was destroyed by Klenow fill in and blunt end ligation. The Xho1-Sal1 herpes simplex thymidine kinase (HSV*tk*) gene was then cloned into the remaining Xho1 site. The Hind3 site 5' of the DQ52 gene was also eliminated in order to allow cloning of the heavy chain gene into the Hind3 site 5' to the HSV*tk* gene. The heavy chain gene was the Sal1-Xho1 fragment from pμ [Grosschedl *et al.* 1984]. It contains the Ig promoter, leader, VDJ, and C_μ excluding the membrane exons. Because the targeting vector is derived from sequences of strain BALB/c, while the ES cell line is derived from strain 129, it was also necessary to assess the extent of DQ52 and C_μ polymorphism between the two strains. ES cell genomic DNA digested with BamH1 and Hind3 was subjected to Southern analysis; no polymorphism was detected with these enzymes.

We verified our t-v.1 construct by restriction analysis and Southern blotting. Hae2, Hind3, and BamH1 digests were compared to a vector with no C_μ and to vector pμ. The blots were probed with C_μ1-3 (cDNA containing C_μ exons 1, 2, and 3) and C_μ3-4 (a 1.2 Kb Hind3 fragment containing exons 3 and 4). The number and size of all fragments were consistent with the sequence of the vector t-v.1 being as planned. Using immunofluorescence and SDS gel electrophoresis, we demonstrated that hybridomas transfected with t-v.1 produce μ H chain. Thus it is probable that if the J_H stretch is replaced by V_H17.2.25DJ_4 in the mouse genome, μ chain containing that V region will be expressed.

Targeted ES cell clones. When we transfected the targeting vector t-v.1 into the embryonic stem (ES) cell line AB-1, we obtained *ca.* 3000 neomycin and gancyclovir doubly resistant colonies in each of three independent transfections. DNA was probed with sequences that were absent in the plasmid and 5' of the genomic sequences to be replaced. After digestion with appropriate enzymes that generated fragments, targeted replacements were indicated by a difference in length of fragments containing both the 5' sequences and J_H. Rehybridizing with a probe containing the *neo*^{r} gene confirmed that there was only one integration site of *neo*^{r}, and its intensity was compatible with there being a single copy. As predicted for a homologous recombination event, the *neo*^{r} probe hybridized with the same band as the 5' probe. By these criteria, one out of 50 transfectants contained a targeted event. Further Southern blot analyses were undertaken with the enzymes BamH1 and EcoR5 and probes containing μ and V_H17.2.25 sequences; these analyses indicated that the introduced rearranged VDJ segment was linked to C_μ and thus likely to be functional. As the plasmid that was used for transfection contained a deletion in the switch region S_μ, we were able to determine that the 3' crossover occurred 5' of S_μ in one ES cell clone and 3' of S_μ in another.

Targeted mice. The first targeted ES cells were tested and determined to be free of known virus and mycoplasma infection before being injected into blastocysts at the DNAX Institute, in collaboration with Richard Murray and Paulo Vieira (see enclosed letter), who have generated several mouse lines with mutations targeted to interleukin genes. We now have the cabability of producing own mice here at UCSF. With the help of Louis Reichardt and Gail Martin, we have injected targeted ES cells into blastocysts of both J_H knockout mice and C57Bl/6 mice and obtained over 30 chimeras. So far we have obtained several mice of various degrees of chimerism, some produced at DNAX and others produced in our lab. The first round of DNAX mice were all females, which, because the ES cells were male and also because the number of eggs is far less than the number of sperm, have little chance of germ line transmission. Now we have several good male chimeras, some resulting from injections into J_H knockout blastocysts, which are being bred now.

As the J_H knockout is not leaky, any B lymphocytes in such mice must be derived solely from the ES cells. H chains will be predominantly, if not exclusively, derived from the V_H17.2.25 replacement transgene. From immunofluorescence staining of peripheral blood cells, we know that V_H17.2.25 is expressed. This protocol will enable us to determine relatively rapidly whether or not an inducible V_H gene functions.

Experimental Design

Generation of the memory replacement mouse

Gene targeting into the germ line is achieved in three steps [Martin 1981; Gregg & Smithies 1986; Robertson *et al.* 1986; Thomas & Capecchi 1986, 1987; Doetschman *et al.* 1987, 1988; Mansour *et al.* 1988; Joyner *et al.* 1989; Thompson *et al.* 1989; Zimmer & Gruss 1989]. First, the appropriate DNA construct is made. Then all or part of the construct is introduced by homologous recombination at the correct site of the genome of a cell of a pluripotent embryonic stem (ES) cell line [Robertson *et al.* 1986]. Then cells of this transfected line are introduced into blastocysts of a mouse with a different genetic background and coat color to produce a mosaic mouse.

Constructing and cloning a tetracycline-responsive Ig H chain gene. The Hind3-Xho1 fragment containing the DQ52 exon and the neomycin resistance gene are isolated and purified from t-v.1. The tetracycline operator sequence, which includes the hCMV minimal promoter sequence, is the Xho1-BamH1 fragment from plasmid puhc13-3, which we have obtained from H. Bujard. The two fragments are ligated and cloned into the Hind3 and BamH1 sites of the modified vector pgp1f. The μ sequences and the HSV*tk* gene for negative selection are taken from the Nde1 fragment purified from t-v.1, which excludes all 5' sequences of the heavy chain promoter up to the initiation site for transcription. This fragment is cloned into the Nde1 site of the vector pgp1f, 3' to the DQ52-neo-tetO sequences to obtain the plasmid pμtet.

An alternative construct lacking the heavy chain enhancer will also be made. The Nde1 fragment containing μ is cleaved with EcoR1. The two resulting fragments are separated and purified. The 5' fragment contains VDJ plus enhancer and is further digested with Nae1. The largest fragment is then isolated; it contains VDJ but not the enhancer. Both this fragment and the 3' fragment of the first digestion, containing C_μ, are blunt ended by Klenow fill in and ligated together. The enhancerless μ gene is then cloned into the EcoR5 site of the Bluescript KS vector (Stratagene). As a Xho1-Not1 fragment it is finally cloned between the Xho1-Not1 sites of the pgp1f vector.

Both constructs will be transfected and tested for inducibility in the hybridoma A12.15 and the fibroblast cell line 3T3. Since the replacement vector contains a functional μ gene, H chain expression can be tested.

Targeted transfection of ES cells. Gene targeting into the ES cell line AB-1, derived from mouse strain 129, is done by microporation. The constructs contain the *neo*^{r} gene for positive selection, *i.e.*, selection for the presence of transfected DNA, and the HSV *tk* gene for negative selection, along with a region of target gene homology. When a double recombination event occurs, one on each side of the *neo*^{r} gene, the HSV *tk* gene will fail to integrate and will be lost. Such an event is most likely to occur during homologous recombination, and cells selected in this way ought to be enriched for targeted mutations. Then the ES cells will be supertransfected with the transactivator vector, selected with hygromycin, and several independent supertransfectants will be cloned.

We have adapted a protocol for preparing DNA directly from 96 well plates in order to screen for homologous recombination by Southern blot analysis. This protocol was reliable and fast for identifying targeted integrations for our "replacement mouse".

Blastocyst injection and breeding of replacement mice. As described above in the supporting data section, we have already injected ES cells with the simple non-inducible VDJ targeted replacement into blastocysts and generated chimeric mice expressing the introduced VDJ segment. The recipient blastocyst is of the C57BL/6 strain, which is black. Chimeric mice that develop from the blastocysts are identified by the presence of patches of colored fur donated by the ES cell line derived from the agouti-colored 129 mouse. These mice are bred to produce F1 progeny, which will contain a proportion of agouti mice; this proportion depends on the extent to which the ES cells contributed to the germ line. About one-quarter of the agouti mice will be heterozygous for the targeted mutation and the transactivator and so may be crossed among themselves to achieve mice that are homozygous for the introduced genes. The variants will be backcrossed to a standard strain to produce a congenic line.

We also plan to inject targeted ES cells into blastocysts of $H^-\kappa^-$ double knockout mice. The embryo will develop into a chimeric mouse with B lymphocytes derived solely from the ES cells. This will allow us to decide early whether or not to proceed with a targeted ES line; moreover, those mice can be used for some memory studies.

Preliminary characterization of the memory replacement mouse

V_H gene replacement. This phenomenon has been observed in transformed cell lines, but it is not thought to be likely to occur at an appreciable rate *in vivo*. To determine whether or not V_H replacement is a *frequent* event, the intensity of V_H17.2.25 hybridization to the specific band containing the VDJ segment will be compared to the intensity of the C_μ band on Southern blots. B cells from various tissues, and, if necessary, hybridomas of LPS-stimulated spleen cells will be tested.

Were V_H replacement to be a major contributor to V region diversity, it would be of considerable interest. But it would also mean that the memory replacement mouse would loose its monoclonality, which could warrant some additional changes in the VDJ transgene construct, *e.g.*, mutating the heptamer sequence in V_H17.2.25.

Anti-NP titers will be determined before and after antigen stimulation of induced and uninduced mice, as will the concentration of all serum Ig isotypes. We will determine whether all of the serum Ig does indeed bind NP. B lymphocytes in bone marrow, spleen, and lymph nodes will be analyzed by flow cytometry with antibodies specific for the H chain classes, for our newly found pre-B cell marker NaKβ1, for the pre-B and B cell marker B220, and for λ L chain . The number of NP-binding B cells will be assessed by flow cytometry with NIP-coupled BSA and FITC-coupled anti-BSA antibody.

Use of the inducible monoclonal mouse for the study of immunological memory

Is antigen required for maintainance of immunological memory? It has been proposed that memory B cells require continual stimulation by antigen. With a monoclonal mouse with its Ig H chain transgene driven from an inducible promoter, we can study this question from the antigen receptor side. After primary immunization, the heavy chain gene will be turned off with the consequence that Ig antigen receptors can no longer be made. The virgin (*i.e.*, non-memory cell) B cell population will presumably disappear. Memory B cells that need continuous antigenic stimulation will also disappear. But those memory B cells that do *not* need continuous antigenic stimulation, if such memory cells exist, ought to survive without receptors. After some time, heavy chain production in the latter type of memory cells will be turned on, which should allow a secondary response induced by antigen. If all memory cells require continuous antigenic stimulation, the response will be primary.

We propose to raise our mice without tetracycline in the drinking water so that immunization with NP-CG will elicit a primary anti-NP immune response, which we will measure by determining antibody titers and enumerating hemolytic plaques every other day for a week after injection. Then the mice will be put on tetracycline, so that they no longer synthesize Ig H chain; hence no new B cells. The memory cells might or might not stay around, which is the critical question we want to ask. After various intervals of exposure to tetracycline, it will be removed in order to allow the synthesis of IgM by newly formed B cells and/or the re-synthesis of IgM by memory B cells that survived the period of tetracycline inhibition. A second injection of NP-CG will be given after reinduction, and the magnitude and kinetics of the response will then be measured again.

After reinduction, we will also sequence the V_H gene segments to distinguish between primary and memory B cell responses. So far hypermutation is the only reliable marker of a memory B cell. There is some mutation in a primary response, but their frequency is much greater in a memory response. Thus, the average number of mutations serves as a means to distinguish between a primary and a memory B cell response. If the average number of mutations per V exon is above a predetermined baseline level, the response can be considered to result, at least in part, from memory B cells. To establish this baseline, we will sequence V exons in B cells (or hybridomas generated therefrom) during a primary response. Three times the standard error will be added to the mean number of mutations per V exon in order to obtain the cutoff value, above which a cell population can be considered to be a memory cell population. Mutations can be scored by amplifying the V_H17.2.25DJ_4 transgene, the only VDJ

present, by polymerase chain reaction (PCR), cloning the products into a sequencing vector, and sequencing individual clones. If we encounter ambiguities with this approach, we will generate hybridomas from the mice and sequence the V_H gene segments by amplifying DNA from the hybridoma cells. As the unmutated sequences are known, we anticipate few problems with PCR. But, as a mutation might destroy the functionality of a PCR primer, at least two different primer pairs will be used.

Characterization of memory B cells. If memory persists through the period of tetracycline treatment even though there can be no stimulation by residual antigen during this period, it will be easy to characterize memory B cells. The isotypes they produce will be determined by flow cytometry. But even if memory does not persist, the monoclonality of memory replacement mouse cells means that most of the switched, IgG^+ B cells will be memory cells. So in either case, we should be able to obtain a reasonably pure memory cell population. It may be important to study the cells that have not switched their immunoglobulin class. Nevertheless, the importance of IgG and other immunoglobulin isotypes in a memory response is indisputable. Indeed, a study of the requirements for the maintenance of IgE memory might be of immense practical importance.

Search for new memory cell markers. While memory B cells are often *defined* to be IgG-producing cells, this definition is simply a matter of technical convenience. In a secondary immune response, all IgG-producing B cells are memory cells, while the set of IgM-producing B cells is composed of both memory cells and naive cells. For further studies of immunological memory, it would be of great importance to have cell surface markers that were specific for memory cell *per se.* Membrane preparations of cells of this population will be repeatedly injected into rats and their spleen will be taken to generate hybridomas. The supernatants of Ig^+ hybridomas will be tested against the memory cells and the naive cells, i.e., spleen cells from mice that were not injected with antigen. In this way, we will try to find monoclonal antibodies to memory cell markers.

Literature cited

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4. LABORATORY SPACE and FACILITIES available to the applicant.
   State location if facilities are elsewhere than the applicant's institution.
   Indicate any facilities that are required but are not currently available.

Dr. Wabl's lab space includes 2300 square feet in bench space and offices. It contains a P2 facility, a dark room, and a sterile room for tissue culture. The lab has access to a large animal facility that is located on the same floor as Dr. Wabl's laboratory, staffed by fulltime animal caretakers. Available equipment items include: Sorvall RC-5G centrifuge, Beckman L2 ultracentrifuge, CO2 incubator, freezers, spectrophotometer, sterilizer, and power supplies. On the same floor there is an autoclave and a dishwasher available to Dr. Wabl's group, as well as the general FACS facility and a Cs source for gamma radiation.

5. BUDGET JUSTIFICATION - Use this space to explain specific needs for items described on budget pages.

Mice: Total for the first yr: **$ 26, 370.** Per diem charges 0.50 $/cage and 0.77 $/cage in conventional and transgenic facilities, respectively; 20 injections to produce a reasonable number of chimeras that give germline transmission. *Strain 1*: F1 of C57Bl/6 and DBA2 females: foster mothers; 80 animals total, housing 40 at a time. Cost (price + shipment) is 650+170 = 820 $; housing 840 $. F1 of C57Bl/6 and DBA2 males: used as vasectomized partners of the foster mothers; 6 animals in total housed one per cage, at 50 $. 6 mice in separate cages, 6 mo, transgenic facility is 840 $. *Strain 2:* C57Bl/6 females: 200 as blastocysts donors. Housing 50 at a time, 6 mo, at 1580 $. C57Bl/6 males: mating partners of the blastocyst donors. 10 animals, 80 $, 6 mo in separate cages is 900 $. *Strain 3:* Double J_H and k knock-out: mating partners and blastocyst donors. 10 breeding cages for long term, at 2800 $/yr; in addition 40 blastocysts donors while performing injections, at 850 $ for 6 mo. As partners for the donors 10 males in separate cages during the injection period, at 1400 $ for 6 mo; 40 cages to keep pregnant and non-breeding mice at 11080 $ /yr. *Special services* charged by the mouse house, incl. sterile equipment and clothings: 1500 $/yr. *Transgenic lines:* For keeping the lines 10 breeding cages permanently, at 2800 $/yr. In addition to the mating cages 30 other cages for mice being studied at 8400 $/yr.

6. APPENDIX: Place the appendix materials after the original and each copy of the application form as indicated in the Instructions for New Applications.

   a. Biographical Sketches of the professional personnel to be associated with the project.
      Each sketch should be NO MORE THAN TWO (2) PAGES. The NIH format is acceptable.
      The P.I. should include and indicate by an asterisk the FIVE (5) most significant publications whether or not they relate directly to this application.
   b. Supporting material (such as letters of collaboration).
   c. Copies of not more than FIVE (5) of the applicant's publications or manuscripts that are pertinent to the project.

7. ABSTRACTS of PUBLICATIONS : *Only one set is required.* See Instructions for New Applications.
   Submit ONE PHOTOCOPY of the abstract page of each "pertinent publication" included in the appendix (6.c.) above;
   For each manuscript, submit a single composite page that includes authors, title, journal, abstract and publication status (for example, "submitted for publication").

P.I. Name Matthias R. Wabl, Ph.D.

7. FIRST YEAR'S BUDGET:

A. Salaries. Give % time even if no salary is requested. State names or "to be recruited".

	% time	Amount
Professional Personnel including Principal Investigator		
Matthias R. Wabl, Ph.D., Principal Investigator	20%	$22,515
Michelle Jumper, Ph.D., Postdoctoral Fellow	100%	$23,334
Technical Support		
Nishay Chitkara, Staff Research Associate	50%	No Salary Requested

A. Salaries Subtotal $45,849

B. Consumable supplies (by major category)

Radioisotopes		$2,000
Tissue culture media, serum, plasticware		$4,500
Chemicals (Enhance,DNA polymerase, PCR kit, oligomers,nick translation		$3,630
Restriction enzymes	$1,900	
Antisera	$500	
X-ray film	$700	
Nitrocellulose	$1,500	

B. Consumables Subtotal $14,730

C. Other Expenses (itemize)

Animal purchases, maintenance		$26,370
Research-related office supplies		$250
Postage, shipping		$250
Slides, photography		$750
FACS analysis		$500
Journals, texts	$250	
Radioactive waste disposal	$750	

C. Other Expenses Subtotal $29,120

A+B+C Subtotal $89,699

D. INDIRECT COSTS **(15% of A + B + C).**

D. Indirect Costs $13,455

E. Permanent Equipment (itemize)

None

E. Permanent Equipment Subtotal 0

Indicate here and on Page 1.

F. ..... TOTAL REQUEST $103,154

Indicate here and on Page 1.

8. PROJECTED BUDGET AMOUNTS:

BUDGET PERIOD	Salaries, Supplies and Other expenses	Permanent Equipment *	Indirect Costs	TOTAL
Year 2	$93,687	None	$14,053	$107,740
Year 3 if applicable	$99,042	- 0* -	$14,856	$113,898

*You may not use CTR funds to purchase permanent equipment in the terminal grant year.

CURRENTLY ACTIVE GRANTS , CONTRACTS and OTHER SOURCES of FUNDS
List financial support (direct costs, only) from all sources, including own institution.

Title of Project	Sources (give grant numbers)	Total Value of Grant (direct costs)	Current Annual Amount Available to You	Date of Termination of Grant
Genetic Variation in B-Lymphocyte Ontogeny	NIH GM37699	$469,300 for 7/94-6/97	$150,403	6/30/97
Catalytic Antibodies for AIDS Therapy	Miles Pharmaceuticals	$200,000	$100,000	6/30/95
Multi-purpose Arthritis and Musculoskeletal Disease Center	NIH AR20684	$100,000	$33,670	12/31/96
Inducible Immunoglobin Transgene Expression	UCSF	$12,500	$12,500	6/30/95

Identify and describe any overlap of this application with the above grants:

No overlap.

Indicate the total annual funds available to you this year for all research projects under your supervision.	$ 296,573

PENDING OR PLANNED

Title of Project	Sources (give grant numbers)	Total Value of Grant (direct costs)	Avg. Annual Amount Available to You	Total Duration (give inclusive dates)
Mice with an inducible monoclonal B cell compartment for studying memory and autoimmune disease	Arthritis Foundation	$208,332 requested	$69,444 requested	7/1/95-6/30/98

Identify and describe any overlap of this application with the above project.

The proposal to the Council for Tobacco Research and the proposal to the Arthritis Foundation are identical. Should both be funded, only one award will be accepted.