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Antigen Specific Induction of T Cell Derived Antigen Binding Molecules

Marianne Murray Urbanski

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ANTIGEN SPECIFIC INDUCTION OF
T CELL DERIVED ANTIGEN BINDING MOLECULES

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INTRODUCTION

Upon interaction with antigen, T and B lymphocytes respond to the stimulus in a highly specific manner. Antigen stimulated B cells proliferate and differentiate into immunoglobulin secreting cells which secrete immunoglobulin specific for the inducing antigen. The antigen combining site of the secreted products is identical to the antigen combining site of the B cell membrane immunoglobulin receptor.

Antigen may also interact with the T cell through the T cell receptor (TcR), and this response is usually dependant on antigen recognition in association with Major Histocompatibility Proteins (MHC). Some investigators have shown, however, that T cells also produce antigen binding molecules (TABM) which bind nominal antigen and are not restricted by MHC glycoproteins. These molecules may be either soluble (sTABM) or membrane-associated (mTABM). The soluble form generally function as effector molecules [1-8] and the membrane form are primarily antigen receptors [9]. The effector function of the TABM may be either to help or to suppress the immune response depending on their structure and association with other proteins [1, 3]. In addition, they may be involved in immunoregulation and hypersensitivity on a systemic level.

Various studies [10, 11] have shown that injection of supernatants of T cells (or a T cell hybridoma) into animals renders them unresponsive to a specific antigen. The factor responsible for this was later shown to be a T-cell antigen specific protein (TABM). Similar experiments have shown TABM
augment: A) antigen specific antibody responses [12, 13], B) DTH response [14] and C) specific CTL responses [15]. In addition, these molecules have been shown to transfer the ability to elicit an immediate hypersensitivity reaction independent of IgE [16].

Various investigators have identified different TABM based on their specific antigen binding. TABM have been isolated from: A) culture media of T cell hybrids [17, 18], B) clones [4, 5, 19] or sensitized T cells [2], C) serum [16, 20] D) ascites fluid of antigen-specific T cell hybrids [21]. These proteins are different from the B cell produced immunoglobulin and the MHC-restricted T cell receptor (TCR). In general, TABM are acidic, hydrophobic and tend to aggregate. The primary structure of TABM appears to be a 110 kD protein which reduces to 72 kD, 45 kD and 25 kD subunits. In addition, a 140 kD form has been isolated which may be a disulfide linked multimer [21].

TABM require an accessory molecule to demonstrate immunoregulatory activity. These additional factors are not antigen specific; they determine the genetic restrictions of the TABM-accessory molecule complex and bear determinants bound by anti-I-J or anti-Ia antibodies [3]. Some evidence has shown that the function of the regulatory factor is determined by the TABM, while the target cell is determined by the accessory molecule [22]. These findings represent a new kind of immune response that involves antigen specific (not MHC associated) T cell produced molecules.
OBJECTIVE

By definition, TABM are proteins that bind antigen specifically. Many investigators have isolated these proteins and have demonstrated their potent immunoregulatory effects. However, no one has demonstrated that formation of TABM in vivo is inducible by a specific antigen. If these proteins can be shown to be inducible in animals upon antigenic stimulation, it will indicate that these cells can be triggered at the site of antigen challenge to execute their functions. Because we already know of the extensive involvement of these molecules in immunoregulation, this indicates great biologic significance. This project attempted to substantiate prior observations that soluble TABM are antigen specific proteins that are secreted by T cells and are different from B cell produced immunoglobulin.

The goal of this investigation, therefore, was to demonstrate that the production of T cell antigen binding molecules (TABM) in mice is inducible upon stimulation with antigen and to demonstrate that this response is antigen specific.

SPECIFIC OBJECTIVES

1) To demonstrate that TABM production in vivo is inducible.
2) To demonstrate that these TABM are antigen specific.
3) To show that these TABM are T cell derived.
LITERATURE REVIEW

GENERAL

Some T cells produce soluble molecules which are not restricted by gene products of the major histocompatibility complex (MHC) in their recognition of antigen [3, 7, 9, 18, 21]. These antigen binding molecules (TABM) may have an effector role in immunoregulation [3-5, 8, 22-25] or may have an affector role in immune responses such as contact sensitivity [1, 2, 16, 26-29]. TABM may also be found associated with the cell membrane where they may function as antigen receptors [9, 21]. Membrane TABM (mTABM) have been found on at least 80% of adult murine thymocytes and they are expressed by day 16 of gestation [30, 31]. In addition, thymic and peripheral mTABM appear to express similar epitopes suggesting similarities between thymic and peripheral mTABM.

The heterodimeric αβ T cell Receptor (TcR), unlike TABM, does not possess this ability to bind to nominal antigen with high affinity; its recognition is dependent on the association of antigen with MHC glycoproteins. It also differs from these soluble products in molecular size [3, 9, 20]. The basic subunit size of TABM is a Mr 22,500 polypeptide which may form multimers of Mr 45,000, 71,000 or 110,000 in the reduced state and they may be seen as Mr 140,000 or larger polypeptides in their unreduced state [2, 3, 11, 17, 27, 29, 32-37]. Multimerization is facilitated by the presence of calcium and reduction to the basic subunit requires treatment with 5M guanidine [38]. The TcR α and β chains are Mr 35,000
and 45,000 respectively [21, 39-42]. Also, unlike TABM which may be found as a soluble or as a membrane bound form, the TcR has only been identified associated with the cell membrane. Amino acid composition, however, suggests some similarity of TABM to TcR α or β [40].

TABM have been isolated from (a) culture media of hybridomas [4] or T cell clones [18, 34, 43], (b) normal and hyperimmune serum in mice [7, 8, 20, 35], (c) normal human serum [44] and d) ascites fluid [20, 38] of antigen-specific T cell hybrids. In addition to detecting TABM in normal mouse sera, Cone et al. [20], also demonstrated TABM in the sera of immunized and desensitized mice. Although a modest (1-2 fold) increase in total TABM levels was obtained after immunization only, a 300 fold increase in total serum TABM was observed within 12 hours of administering a desensitizing dose of antigen into primed mice. The TABM detected was both specific for the desensitizing antigen and antigen nonspecific suggesting both an antigen specific and nonspecific component to desensitization. Ferguson and Iverson, [7] hyperimmunized mice to SRBC or HRBC and demonstrated production of the antigen binding portion of the two chain T suppressor inducer factor (TABM) in the serum using a monoclonal antibody to murine TABM. This molecule was dependent on an antigen-nonspecific co-factor for suppressor activity. Finally, DiBrino et al. [44] isolated TABM from normal human sera using procedures that have been developed to isolate murine TABM. The human TABM demonstrated α mobility in immunoelectrophoresis as do the antigen-specific molecules of mice, and it represented 0.02%-0.05% of the total serum protein. Also, the antigen binding molecules identified were bound by an antiserum made against a
synthetic peptide corresponding to the J region of the TcR β chain which suggested a possible homology between the human serum protein and the TcR β chain.

In general, polyclonal TABM are heterogeneous in charge, whereas monoclonal TABM exhibit charge homogeneity [33]. Their isoelectric point range is between 4.9-7.2 depending on the specific TABM. TABM are insoluble in water and precipitate with 43-50 percent (NH₄)_2SO₄ indicating that these molecules are globulins. There is evidence that, like immunoglobulins, common antigenic determinants exist on TABM [45]. Peptide mapping using *Staphylococcus* V8 protease and amino acid analysis have revealed similarities and distinctions between different antigen specific TABM, which suggests constant and variable regions of the molecules. Also, several heteroantisera to murine or rat TABM prepared against different antigens bound to many of the TABM. This represents a "constant region" for binding by the T cell secreted products. However, these proteins are clearly T cell secreted [1, 3-10, 12, 16, 20-22, 25-28, 32, 33, 38-40, 44-52] and therefore differ from B-cell secreted immunoglobulins.

FUNCTIONS OF SOLUBLE TABM

A) Immunoregulation

The activity of soluble TABM depends on their structure and association with other proteins [3, 6, 18, 23, 43]. These accessory molecules (NAgBC) do not bind antigen; they bear serologic Ia or I-J determinants and determine the genetic restriction required for interaction with target cells [22,
The TABM (AgBC) combined with the NAgBC comprise the T suppressor (TsF) and T helper (ThF) factors.

The action of the T cell suppressor circuit will ultimately result in suppression of T-helper activity and involves a complex set of T cell interactions [53]. Suppression begins by activating a suppressor inducer population of T cells. These inducer cells are CD4+,CD8-, I-J+ [6, 8] and are responsible for producing the TsIF (T suppressor inducer factor) which is composed of an antigen binding, (AgBC) chain and a non-antigen binding (NAgBC) chain [23, 46]. This group of cells then "induces" the transducer cells (TTrans), which are CD4+,CD8-, I-J+. Communication between the inducer cells and the transducer cells is restricted by gene products of the IgVh region of chromosome 12 (in the mouse) [54]. The transducer cells communicate with a T suppressor effector cell which is CD4+,CD8-, I-J-, and produces a T suppressor effector factor (TsEF). This factor contains an antigen binding chain only, but will induce production of the appropriate nonantigen-binding, I-J+ chain from a CD4+,CD8-, I-J+ cell. In the murine model, this interaction between the two chains is restricted by gene products of the I-E region of the MHC, while the biologic action of this complex is restricted by the IgVh region of chromosome 12. [8] The final result of the TsEF is suppression of helper T (TH) cells.

Flood, et al. [6] combined the AgBC from a Ts inducer factor (TsIF) with an I-J+, NAgBC from F12, a T cell hybridoma that secretes an Azobenzene arsonate (ABA)-specific TsF, and produced a fully functional T suppressor factor. This factor demonstrated an Igh-V-linked genetic restriction in its activity, apparently controlled by the NAgBC, I-J+ element.
The genotype of the cell that produced the AgBC was irrelevant; the Igh haplotype of the cell that produced the NAgBC was the restricting element.

In a similar series of experiments in which hybrid AgBC-NAgBC complexes were produced, Flood et al. [22] demonstrated that the function of the regulatory complex is determined by the AgBC and the target cell is determined by the NAgBC. The authors combined the AgBC of the TslF with the NAgBC from a TsEF and demonstrated suppression of the T-helper (TH) dependent function. Similarly, when the AgBC of the TsEF was combined with the NAgBC from a TsIF, induction of suppression (via action on the TTrans) was demonstrated and no effect on the TH was observed. These findings are consistent with their model in that the TsIF delivers the message to the TTrans to induce suppression while the TsEF delivers its message to the TH cell to suppress T-cell help. Therefore, the NAgBC must interact with an Igh-linked product on the appropriate target cell while the AgBC binds the appropriate Ag to deliver the specific signal to the cell.

Many other investigators have substantiated these results by demonstrating that two disulfide linked molecules are required for suppressor function: one antigen specific, I-J- and another antigen nonspecific, I-J+ [4, 5, 8, 23, 43, 46, 55]. Also, the genetic restriction for the Igh linked determinants of the TsF is contained in the I-J+ portion while the antigen specificity is contained in the I-J- portion [6, 22, 23, 46]. Lowy et al. [43] analyzed a suppressor factor secreted from an ABA specific Ts hybridoma and a SRBC factor by separating the NAgBC from the AgBC and recombining them to form hybrid TsF. They demonstrated that an ABA I-J- /SRBC I-J+ hybrid factor suppressed an ABA but not a SRBC response.
whereas an ABA I-J+/SRBC I-J⁻ hybrid suppressed only a SRBC response. Similarly, when an I-J⁺ chain of B10.A (Igh-1ᵇ) mice was added to an I-J⁻ chain from A/J (Igh-1ᵉ) mice, the resulting complex could suppress the cell mediated response of the B10.A mice but not of the A/J mice. Because the mice possess different Igh-1 allotypes, the authors conclude that the I-J⁺ portion of the TsF is critical in determining the genetic restriction of the message.

The immunoregulatory circuit involved in suppression of delayed type hypersensitivity (DTH) is slightly different [8]. CD4⁺,CD8⁻ T cells from mice exposed topically to antigen produce an AgBC and CD4⁻,CD8⁺ T cells exposed to the antigen produce a NAgBC. Neither chain alone will suppress the transfer of DTH, although when combined these chains are able to suppress the transfer of DTH in an antigen-specific manner. When exposed to specific antigen, the AgBC from the CD4⁺,CD8⁻ T cell induces production of a NAgBC by an CD4⁻,CD8⁺, I-J⁺ T cell.

Little, et al. [56] identified a T-helper factor (T HF) which had the ability to augment contact sensitivity in mice. The factor consisted of two disulfide bonded chains: one antigen binding and one non-antigen binding. The antigen binding chain was responsible for the specificity of the complex and the non antigen binding chain was responsible for controlling the activity of the complex in an I-A restricted manner. Thus, reconstitution of the two chains of the T HF requires that the sources be matched at the I-A region of the MHC. This is similar to the TsF subunits which must be matched at the I-J region.
B) TABM in Immune Responses

Some TABM have the ability to transfer contact sensitivity in a manner similar to that of immediate type hypersensitivity [2, 16, 27, 28, 52]. In the classical hypersensitivity reaction IgE binds to mast cells through their Fc receptor. Upon binding to allergen (antigen), the mast cell degranulates causing release of biologic mediators (including histamine) responsible for the sequelae observed in contact sensitivity. Askenase, et al. [27] obtained an antigen specific factor from T cells and injected it into mice to demonstrate an antigen specific immediate hypersensitivity like reaction. This response was independent of IgE but was dependent on the presence of mast cells. The authors propose that TABM, like IgE, bind to the mast cell and cause release of mediators when exposed to antigen. The TABM response is thought to be due to the release of serotonin rather than histamine because the response is inhibited by treatment with reserpine, a monoamine-depleting drug that depletes serotonin from mast cell granules [57]. In general, the response seen with TABM is slightly less intense and shorter acting than the IgE response.

In an attempt to determine if the TABM and IgE bind to the same receptor, Kraeuter-Kops, et al. [28] used an in vitro indirect rosette assay to demonstrate that antigen specific factors (TABM) interact with the mast cell surface. The authors combined hapten-conjugated sheep red blood cells (SRBC) and mouse peritoneal mast cells and added antigen specific factors, IgE or IgG. This resulted in formation of rosettes, and this response was antigen specific. Antigen specific rosette formation was detected in the presence of nanogram quantities of TABM while more than 10 times greater
amounts of IgE were needed to produce IgE-mediated rosettes. Removal of the TABM from the system removed the ability to form TABM rosettes and removal of IgE blocked the ability to form IgE rosettes. Similarly, preincubation of mast cells with IgE of various specificities, but not other immunoglobulin isotypes, blocked IgE rosettes but not TABM-induced rosettes, and preincubation of mast cells with antigen blocked antigen specific rosette formation but not IgE rosette formation. These results suggest that the TABM interacts with a unique receptor on the surface of the mast cell in the mouse.

Helfgott, et al. [58] have injected a collagen-specific molecule from a collagen specific T-cell clone into the synovia into rats and induced arthritis when exposed to antigen. This arthritogenic factor (AF) was a 65 kD protein and has also been isolated from rats with adjuvant-induced arthritis [37]. The synovitis was apparent within 48 hours of injection, arguing against the possibility of B-cell recruitment or antibody production. Also, addition of cobra venom factor did not alter the response, suggesting the response is independent of complement. Thus, it appears that in addition to their potent role in immunoregulation, some TABM are also involved in the immune response.

**TABM AND THE T CELL RECEPTOR**

Several investigators have recently studied the apparent association between the TcR and TABM [40-42, 51, 59-62]. Moorehead, et al. [51] observed that an anti-TcR antibody binds to an antigen specific T suppressor
factor and concluded that the TcR and TABM at least share serologic determinants. Similarly, Schluter and Marchalonis [63] prepared a synthetic peptide that corresponds to the J region of the TcR β gene. Antibodies to this peptide bound to human, dog and murine IgG (particularly to κ light chain) and also bound to a murine suppressor cell. Hubbard et al. [40] have also shown that TABM bear the Jβ determinant, thus supporting the contention that TABM and the TcR share serologic determinants. Guy et al. [25] hypothesized that the antigen specific helper factor actually represents a soluble form of the TcR. Their helper factor was a disulfide-linked dimer that was precipitated from cell-free supernatants of cloned TH cells with a monoclonal antibody specific for the TcR Vβ8 determinant.

Many problems arise when thinking of TABM as a soluble form of the TcR. Firstly, TABM do not need MHC to recognize antigen, and the TcR must "see" antigen and MHC together in order to respond. Although there have been reports of the TcR binding nominal antigen [64] the binding was with extremely low affinity. Also, the genes that code for the β chain of the TcR are not always present in the T cell hybridomas that produce TABM [65]. It is a possibility that the β chain is responsible for the MHC restriction and the accessory molecule, and the α chain is involved in the TABM and antigen recognition.

The relationship between TABM and the TcR was also examined through the use of antisense oligodeoxynucleotides [19, 66]. Using an antigen-specific factor derived from a TH hybridoma, A1.1, the authors showed that antisense corresponding to the Vα oligodeoxynucleotides inhibited CD3 expression on trypsin treated cells and also inhibited production of the antigen
specific factor. Factor production was restored after removal of antisense Vα. Cells treated with antisense to TcR α without prior trypsin treatment expressed normal levels of cell-surface CD3 and TcRαβ but were unable to produce the factor. Also, cells treated with antisense corresponding to Vβ did not produce CD3 but did produce the factor. This indicates that cell surface TcRα is involved in production of the factor, but it probably is not the source of the factor [66]. More recently, Green et al. [19] used retroviral vectors to transfer the TcRα and/or β cDNA from A1.1 to other cell lines and demonstrated that cells expressing A1.1 TcRα, with or without A1.1 TcRβ, produced the antigen specific factor. The authors concluded that the TcR α chain gene from A1.1 confers the ability to produce the factor on other T cell lines, including lines incapable of expressing cell-surface TcR.

In a similar experiment to that of Flood et al. [22], Fairchild et al. [61] separated the NAgBC from the AgBC and observed that the AgBC expressed an epitope bound by a mAb specific for a TcR α-chain-constant region determinant. Also, the NAgBC expressed an epitope bound by an anti-Vβ8 mAb. By forming hybrid complexes using chains of either Kd or Dd restriction, the authors observed that the NAgBC was responsible for the MHC restriction while the AgBC was responsible for the antigen specificity for the functioning complex. Their results suggest that the TcR α chain may be related to the TABM and the TcR β chain may be related to the accessory molecule.

The difference still remains however in molecular size between the Vα and TABM. As stated earlier, TABM is composed of multimers of a 22.5 kD soluble molecule while the α chain has only been identified as a 35 kD membrane bound molecule. In addition, preliminary sequencing using a Ts
cDNA library and a panel of monoclonal antibodies as probes, Kraig has determined that the cDNA for the antigen receptors on the Ts cells does not correspond to any known Ig or TcR gene [67]. Future investigations may find that the membrane bound TcR may be cleaved and/or altered in such a way that it would resemble soluble TABM. The fact remains, however, that in its membrane bound state the TcR and TABM represent different entities and they must be treated as such.
MATERIALS AND METHODS

ANIMALS

BALB/c mice approximately 6-8 weeks old were obtained from Charles River Breeders (Wilmington, MA) or Harlan Laboratories (Indianapolis, IN). C.B. 17-\textit{scid} mice were obtained from the breeding colony of Dr. Leonard Schultz, Jackson Laboratory. Because of their immunodeficiency, the \textit{scid} mice were maintained on a combination of sulfamethoxazole and trimethoprim. All animals were maintained by the Center for Laboratory Animals at the University of Connecticut.

ANTI-TABM

Rabbit anti-mouse TABM that has been previously prepared in our laboratory was used [33]. In brief, rabbits were immunized by subcutaneous injection of 25\(\mu\)g of Mr 110,000 TABM specific for Azobenzene arsonate (TABM-ABA) in complete Freund's adjuvant. Two weeks later they were injected with another 25 \(\mu\)g TABM-ABA in Freund's incomplete adjuvant. Two weeks following this they were challenged by intravenous injection of 50\(\mu\)g TABM-ABA in 300 \(\mu\)g polyadenylic:polyuridylic acid (Poly A:U, Boehringer-Ingelheim, Danbury, CT). Rabbits were bledd at least 7-10 days after the final boost. The antiserum was adsorbed with sepharose beads conjugated with murine Ig or albumin and was screened in ELISA for binding specificity to TABM. The antiserum binds the constant region of secreted TABM [45] but does not bind immunoglobulin or murine serum albumin.
IMMUNIZATION

BALB/c mice were immunized by intraperitoneal injection of 500 μg ovalbumin (OVA), hen egg lysozyme (LYS), or bovine serum albumin (BSA) (all obtained from Sigma) as antigens combined with polyadenylic polyuridylic acid (Poly A:U) adjuvant. Some of the mice were boosted 2 weeks later with another 500 μg of antigen in sterile PBS. The mice were bled from the retroorbital venous plexus at various days up to 21 days post-immunization. The serum was aliquoted and maintained at 4°C or -20°C until it was used. Some mice were immunized with different amounts of antigen ranging from 5 μg to 15 mg combined with 300 μg poly (A:U). The mice were then bled as before on day 14 and the responses for TABM and immunoglobulin production were compared. Scid mice were immunized as described above or received a suspension of 5 x 10^7 BALB/c thymocytes in sterile PBS administered iv, and two weeks later were injected i.p. with 500 μg BSA combined with 300 μg poly (A:U). Two weeks later the mice were challenged with 500 μg BSA in sterile PBS. The serum was collected 14 days after the challenge and TABM and immunoglobulin production were monitored by ELISA.

THYMOCYTE PREPARATION

Thymocytes for reconstitution of the scid mice were obtained from nonimmunized 6 week old BALB/c mice. The mice were euthanized using an ether chamber in a hood and the thymi were removed and placed in sterile PBS. A cell suspension was obtained by gently extruding the thymocytes from
the capsule with injection of sterile PBS into the thymus. The thymocytes were assessed for viability using trypan blue exclusion. Fifty million thymocytes were suspended in 0.5 ml sterile PBS and administered iv to the scid mice.

ELISA

Assays were performed using ELISA trays coated with 1 μg/well of specific antigen. Coating was done in coating buffer consisting of 0.1M NaHCO₃ made pH 9.25 with Na₂CO₃. The trays remained at room temperature overnight. The trays were blocked with 200 μl 0.1% BSA or OVA per well and incubated for 1 hour at 37° C. The trays were washed five times between all steps with wash buffer (10mM K₂HPO₄, 0.1% Triton X 100 and 0.85% NaCl made pH 7.4 with KH₂PO₄). Dilutions of immunized mouse serum (ranging from 1:100 to 1:14) were added at 100 μl/well and again the trays were incubated for 1 1/2 hours at 37° C. After washing, 100 μl/well of rabbit anti-mouse TABM (R33), rabbit anti-mouse immunoglobulin or normal rabbit serum (NRS) at a dilution of 1:500 was added and incubated as above. Anti-BSA was added to wells without serum as a positive control. Then, 100 μl/well of 1:1000 alkaline phosphatase conjugated goat anti-rabbit IgG (TAGO, Burlingame, CA) was added and incubated for 1 1/2 hours at 37° C. Finally, 100 μl of 1 mg/ml p-nitrophenylphosphate in substrate buffer (105 mg/ml diethanolamine and 10 μg/ml MgCl₂ made pH 9.8) was added to each well. The trays were incubated until an OD of 1.5 was obtained for the positive control. Absorbance was monitored at 410 nM.
PURIFICATION OF TABM BY AFFINITY FOR ANTIGEN

BALB/c mice were immunized with BSA/poly (A:U) and challenged with BSA as described above. Fourteen days after challenge, the mice were bled and the sera was pooled. One ml of sera (diluted 1:2) was mixed with 1 ml sepharose beads conjugated with BSA or OVA at 5 mg protein/ml beads. The beads were washed 3 times with PBS and eluted with 1 ml 2x SDS sample buffer. One ml of the eluate was reduced with 6% β-mercaptoethanol and alkylated with 7%(vol/vol) iodoacetamide. Four μl of this eluate was resolved by SDS-polyacrylamide electrophoresis (PhastGel, Phastsystem; Pharmacia) and a Western Blot analysis was performed to identify the antigen specific TABM and immunoglobulins.

IMMUNOBLOT ANALYSIS

The sample was resolved by SDS-polyacrylamide electrophoresis as described above and the gel was transferred to nitrocellulose paper (Immobilon-P) using wet electrophoretic elution. After the filter was rinsed several times with PBS, it was blocked with 0.5% Tween (polyoxyethylene-sorbitanmonolaurate) and incubated at room temperature for 1 hour. Next, rabbit anti-mouse TABM was added at a dilution of 1:400 and this was incubated overnight at 4°C. Following another wash, alkaline phosphatase-conjugated goat anti-rabbit IgG (1:1000) was added and again the paper was incubated for 2 hours at 4°C. Finally, substrate (30%H₂O₂, 3 mg/ml chloronaphthol in 100% methanol, and tris buffered saline) was added and color change was monitored. The reaction was stopped with cold water after 5 minutes.
DATA ANALYSIS

For each experiment the average and standard deviation were determined from 3 data points. Statistical significance was determined using the students t-test or 2 way Analysis of Variance (ANOVA).
RESULTS

DETECTION OF TABM IN THE HUMORAL IMMUNE RESPONSE

To measure a humoral immune response, mice were immunized with 500μg BSA /300μg poly (A:U) and challenged after two weeks with another dose of antigen in sterile PBS. Sera were obtained prior to immunization and on days 3,7,10,14,18, and 21 after primary immunization or secondary challenge and were tested in ELISA for BSA-specific TABM or immunoglobulin. The kinetics of the serum TABM and immunoglobulin for the primary and secondary response was examined. The average of three data points was used to obtain a value and standard error for each day at each dilution. The average value obtained for the preimmunized control sera was subtracted from each experimental average to determine the net value for each day (see Table 1).

Figure 1A is representative of the primary response for TABM production. TABM specific for BSA were detected by day 7 and peak levels did not appear until day 18 after which time the levels decreased (Figure 1A). Figure 1B shows that anti-BSA-immunoglobulin was detected 7 days after immunization, increased slightly by day 10, and remained stable 21 days after immunization. In comparison, Figures 2A and B show the secondary responses for BSA-specific TABM and immunoglobulin respectively.

This data is summarized in Figures 3 A and B. The titer for each day was determined as the minimum amount of serum (or dilution) required to
achieve an OD reading of 0.2. These figures represent the results of three groups of animals and three experiments performed on each group. In general, the TABM response after the challenge was stronger, peaked concurrent with immunoglobulin, and began to decline while the immunoglobulin titer remained stable. This demonstrates that the TABM kinetics differed temporally from antigen-induced immunoglobulin production.

STORAGE OF SERA FOR TABM

In order to determine optimal storage conditions for TABM the collected sera were maintained at 4°C or -20°C. Because TABM are globulins and tend to stick to glassware and plastic containers, especially when they are repeatedly frozen and thawed [33, 49], the serum was aliquoted prior to freezing. Figure 4 shows the results of an ELISA using serum that had been in the refrigerator for 10 days prior to testing compared to sera that was frozen immediately after obtaining it. TABM were identified with R33. The sera stored in the refrigerator lost all activity while the sera stored in the freezer maintained high TABM levels.
DOSE RESPONSE OF TABM AND IMMUNOGLOBULIN PRODUCTION

In order to study the dose response of TABM and immunoglobulin production, various amounts of antigen were administered to the mice and the sera were collected on day 14. Figure 5A is representative of the dose response for TABM production. TABM were produced with an antigen dose of 50 µg BSA and the response slowly rose with increasing amounts of antigen, peaked at 10 mg, and decreased by 15 mg. Figure 5B is representative of the dose response for immunoglobulin production. In this experiment, immunoglobulin were produced by 50 µg and the titer did not decrease until 10-15 mg.

This data is summarized in Figure 5C. The titer for each antigen dose was determined as the minimum amount of serum (or dilution) required to achieve an OD reading of 0.2. This figure represents the results of two groups of animals and three experiments performed on each group. In general, the immunoglobulin titer peaked at a much smaller dose than TABM and decreased as the TABM titer increased. By 15 mg both TABM and immunoglobulin titers had decreased. This data again demonstrate that the TABM response is different that that of immunoglobulin.

ANTIGEN SPECIFICITY OF TABM PRODUCTION

In order to demonstrate antigen specificity of the TABM, mice were immunized with either BSA or LYS and the sera were collected on day 14 after immunization. The sera were then tested in ELISA for binding to either
BSA or LYS. Figure 6A shows the results of BSA-immunized sera tested against BSA or LYS coated plates and figure 6B shows the results of LYS-immunized sera tested against BSA or LYS coated plates. The BSA-immunized sera only bound to the BSA while the LYS sera only bound to the LYS coated plates.

Antigen specificity was further investigated by performing an immunoblot analysis on serum proteins purified by affinity for antigen. The sera from BSA immunized mice were collected 14 days after antigenic challenge and adsorbed to either BSA or OVA-sepharose beads. The sera were eluted with SDS polyacrylamide gel sample buffer to remove adsorbed proteins. The eluted proteins were detected by SDS-PAGE and immunoblotting with anti-TABM or anti-immunoglobulin. As shown in figure 7A, BSA specific TABM of Mr 110,000 were detected in the eluate from the BSA-sepharose beads while no TABM were detected in the eluate from the OVA-sepharose. TABM were also detected in the effluent from BSA and OVA-sepharose beads. BSA-specific immunoglobulins were also detected in the eluate of the BSA-sepharose (Figure 7B). Proteins eluted from the BSA-sepharose were resolved after reduction at Mr 84,000, 47,000 and 25,000 which are consistent with immunoglobulin μ,γ, and light chains. Immunoglobulin was also detected in the effluent from both the BSA and OVA-sepharose beads but no immunoglobulin was detected in the eluate of the OVA-sepharose. These results demonstrate that TABM (and immunoglobulin) produced upon stimulation with BSA bind only to BSA and do not bind to LYS or OVA.
DETERMINATION THAT TABM ARE T CELL DERIVED

To further document the cell origin of the TABM produced, immunodeficient scid mice were reconstituted with thymocytes (to reduce the possibility of contamination with B lymphocytes). Prior to treatment the mice were anesthetized and bled through the retroorbital venous plexus. The sera were tested in ELISA with anti-Ig to confirm that the scid mice were not producing endogenous immunoglobulin. Any scid determined to be "leaky" based on this assay was not used in any further experiments. The mice were primed with BSA/poly(A:U) and challenged with BSA and the sera were collected on day 14 after challenge. Figure 8A shows that unreconstituted scid mice failed to produce BSA-specific TABM compared to the BALB/c control (p<0.01) (Figure 8B). However, after reconstitution with thymocytes, the scid mice produced an amount of BSA-specific TABM consistent with that of the BALB/c control (p>0.01). The reconstituted mice did not, however, produce immunoglobulin specific for BSA (p<0.01) (Figure 8C).
Table 1

Sample Calculation for TABM Production

Sera was collected on day 0 (pre-immunized) and on various days post-immunization. For each day the average value was determined from 3 data points in ELISA. The average for the preimmunized control was subtracted from each experimental average to determine the net average for each day. Normal rabbit serum control is also shown.

<table>
<thead>
<tr>
<th>µL SERUM</th>
<th>DAY 0</th>
<th>DAY 14</th>
<th>DAY 14 NET</th>
<th>DAY 14</th>
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<td>1.0</td>
<td>.138</td>
<td>.265</td>
<td>.127</td>
<td>.104</td>
</tr>
<tr>
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<td>.828</td>
<td>.469</td>
<td>.132</td>
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<td>7.0</td>
<td>.516</td>
<td>1.112</td>
<td>.596</td>
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</table>
BALB/c mice were immunized with BSA/poly (A:U). Sera were collected prior to immunization and on days 3, 7, 10, 14, 18, and 21 post-immunization and tested for BSA-specific TABM in ELISA. This graph is representative of the data used to determine titers for TABM production in the primary response. Each point represents the net average of three data points after the control average was subtracted.
Figure 1B: Immunoglobulin Production in the Primary Response  
BALB/c mice were immunized with BSA. Sera were collected prior to immunization and on days 3, 7, 10, 14, 18, and 21 days post-immunization and tested for BSA-specific immunoglobulin in ELISA. This graph is representative of the data used to determine titers for immunoglobulin production in the primary response. Each point represents the net average of three data points after the control average was subtracted.
Figure 2A: TABM Production in the Secondary Response  BALB/c mice were immunized with BSA/poly (A:U) and challenged with BSA. Sera were collected prior to immunization and on days 3, 7, 10, 14, 18 and 22 days post-challenge and tested for BSA-specific TABM in ELISA. This graph is representative of the data used to determine titers for TABM production in the secondary response. Each point represents the net average of three data points after the control average was subtracted.
Figure 2B: Immunoglobulin Production in the Secondary Response
BALB/c mice were immunized and challenged with BSA/poly (A:U). Sera were collected prior to immunization and on days 3, 7, 10, 14, 18 and 21 days post-immunization and tested for BSA-specific immunoglobulin in ELISA. This graph is representative of the data used to determine titers for immunoglobulin production in the secondary response. Each point represents the net average of three data points after the control average was subtracted.
Figure 3A: Primary Response: TABM and Immunoglobulin. BALB/c mice were immunized with BSA/poly (A:U). Sera were obtained at the indicated times and tested in ELISA for binding to BSA. Titers were determined as the amount of serum/100μl for TABM or dilution of serum for immunoglobulin required to achieve an OD of 0.2 after the control was subtracted. The data represent the averages of three groups of animals and three ELISA experiments/group.
Figure 3B: Secondary Response: TABM and Immunoglobulin BALB/c mice were immunized with BSA/poly (A:U) and challenged with BSA. Sera were obtained at the indicated times and tested in ELISA for binding to BSA. The titers were defined as the amount of serum/100μl for TABM or the dilution of serum for immunoglobulin required to achieve an OD of 0.2 after the control was subtracted. The data represents the average of three groups of animals and three ELISA experiments/group.
**Figure 4: Storage of Sera for TABM**  BALB/c mice were immunized with BSA/poly (A:U) and the sera were collected 14 days later and stored in the refrigerator or the freezer for 10 days. The BSA-specific TABM were identified using R33. Each point represents the net average of three data points after the control average was subtracted.
Figure 5A: Dose Response of TABM Production

BALB/c mice were immunized with 50, 500, 5000, 10000 or 15000 µg of BSA combined with 300 µg poly (A:U). Sera were obtained on day 14 after immunization and tested in ELISA for TABM binding to BSA. This graph is representative of the data used to determine titers for TABM production. Each point represents the net average of three data points after the control average was subtracted.
Figure 5B: Dose Response of Immunoglobulin Production  

BALB/c mice were immunized with 50, 500, 5000, 10000 or 15000 µg of BSA combined with 300 µg poly (A:U). Sera were obtained on day 14 after immunization and tested in ELISA for immunoglobulin binding to BSA. This graph is representative of the data used to determine titers for TABM production. Each point represents the net average of three data points after the control average was subtracted.
Figure 5C: Dose Response of TABM and Immunoglobulin Production

BALB/c mice were immunized with 5, 50, 500, 5000, 10000 or 15000 μg of BSA combined with 300μg poly (A:U). Sera were obtained on day 14 after immunization and tested in ELISA for binding to BSA. TABM specific for BSA was identified using R33 and immunoglobulin specific for BSA was identified with rabbit anti-IgG (heavy and light chains). The titers were defined as the amount of serum/100μl for TABM or the dilution of serum for immunoglobulin required to achieve an OD of 0.2 after the control was subtracted. The data represents the average of two groups of animals and three ELISA experiments/group.
Figure 6A: Antigen Specificity of TABM: Immunized with BSA

BALB/c mice were immunized with 500µg BSA/poly (A:U) and the sera were obtained on day 14 after immunization. Sera were tested for binding to BSA or LYS coated plates in ELISA. Each point represents the net average of three data points after the preimmunized control average was subtracted.
Figure 6B: Antigen Specificity of TABM: Immunized with LYS. BALB/c mice were immunized with 500μg LYS/poly (A:U) and the sera were obtained on day 14 after immunization. Sera were tested for binding to BSA or LYS coated plates in ELISA. Each point represents the net average of three data points after the preimmunized control average was subtracted.
Figure 7A: Immunoblot of TABM Specific for BSA  BALB/c mice were immunized with BSA/poly (A:U) and were challenged with BSA 14 days later. The mice were bled 14 days after the challenge and the serum was collected. The serum was mixed with BSA-sepharose or OVA-sepharose beads and bound proteins were eluted with SDS sample buffer. The eluted proteins were reduced, alkylated, resolved in 10-15% polyacrylamide gels and transferred to immobilon. The blotted proteins were detected with anti-TABM (R33). Lane A: Proteins eluted from BSA-sepharose; Lane B: Effluent from BSA-sepharose; Lane C: molecular weight standards; Lane D: Proteins eluted from OVA-sepharose; Lane E: Effluent from OVA-sepharose.
Figure 7B: Immunoblot of Immunoglobulin Specific for BSA  
BALB/c mice were immunized with BSA/poly(A:U) and were challenged with BSA 14 days later. The mice were bled 14 days after the challenge and the serum was collected. The serum was mixed with BSA-sepharose or OVA-sepharose beads and bound proteins were eluted with SDS sample buffer. The eluted proteins were reduced, alkylated, resolved in 10-15% polyacrylamide gels and transferred to immobilon. The blotted proteins were detected with anti-immunoglobulin.  
Lane A: Proteins eluted from BSA-sepharose; Lane B: Effluent from BSA-sepharose; Lane C: molecular weight standards; Lane D: Proteins eluted from OVA-sepharose; Lane E: Effluent from OVA-sepharose.
Figure 8A: Production of TABM in Unreconstituted scid Mice  

*Scid* mice were immunized with BSA/poly (A:U) and challenged with BSA 2 weeks later. Sera were collected on day 14 after the challenge. BSA-specific TABM (using R33) were identified in ELISA and are expressed as the OD given by 7μl serum/100μl buffer. The results are the mean ± SE of 6 mice.
Figure 8B: Production of TABM in Reconstituted scid Mice. Scid mice were reconstituted with 5x10^7 BALB/c thymocytes, and were immunized with BSA/poly (A:U) 2 weeks later. They were challenged with BSA 2 weeks after the primary immunization and sera were collected on day 14 after the challenge. BSA-specific TABM (using R33) were identified in ELISA and are expressed as the OD given by 7μl serum/100μl buffer. The results are the mean ± SE of 6 mice.
Figure 8C: Production of Immunoglobulin in Reconstituted scid Mice. Scid mice were reconstituted with $5 \times 10^7$ BALB/c thymocytes, and were immunized with BSA/poly (A:U) 2 weeks later. They were challenged with BSA 2 weeks after the primary immunization and sera were collected on day 14 after the challenge. BSA-specific immunoglobulin (using anti-Ig) were identified in ELISA and are expressed as the OD given with a dilution of 1:4000. The results are the mean ± SE of 6 mice.
DISCUSSION

TABM have been identified in T cell culture media [5, 18, 34] or ascites fluid [20, 38] for many years. They also have been reported in both normal and hyperimmune serum [7, 8, 20, 35, 44]. TABM represent a soluble antigen-specific effector mechanism of regulatory T lymphocytes and participate in immunoregulatory activities [2, 7, 14, 15, 34, 52]. We reasoned, therefore, that they may be induced to appear as soluble products in an immune response similar to B cell produced antigen specific immunoglobulins. We have used an ELISA-based antigen binding assay to detect antigen-specific TABM because of the extreme sensitivity of the assay and its dependence on primary binding. This assay will detect as little as 100 ng TABM, and TABM will bind (nominal) antigen in the solid or liquid phase [34, 38, 45, 48].

An immune response is characterized by: 1) inducibility; 2) specificity and 3) memory. We have demonstrated that TABM production fulfill all of these criteria. The TABM response is inducible because TABM that bind the immunizing antigen only rise during the immune response. Also, a second challenge with antigen resulted in a stronger response only to the primary antigen. Thus, the TABM response was inducible, specific and demonstrated memory.

We have used a heteroantisera to TABM (R33) that was previously prepared in our laboratory. This antisera does not bind immunoglobulins or murine albumin [49] indicating that TABM are not B cell-derived.
immunoglobulins or a common serum protein. In addition, our data clearly show that the TABM response is temporally distinct from that of immunoglobulin after antigenic stimulation. The trend in six experiments was that TABM levels in the primary response peaked two days later and declined sooner than immunoglobulins. The secondary response showed that TABM production occurred concurrently with immunoglobulin production, but the TABM titer decreased while the immunoglobulin titer remained stable. This evanescent TABM response appears to be characteristic and provided further evidence that TABM are not B cell-derived immunoglobulins.

TABM production also differed from immunoglobulin production at different antigen doses. TABM production peaked at 10 mg BSA and then decreased, while immunoglobulin production peaked by 50 μg, plateaued through 5 mg, but then decreased. Perhaps the rise in TABM correlates with an inhibition of immunoglobulin production at extremely high antigen doses.

Immunoblots of TABM bound and eluted from antigen further substantiated that TABM are not B cell derived immunoglobulins. In the reduced form, R33 identified TABM which were resolved as Mr 110,000 polypeptides. Anti-immunoglobulin, however, identified polypeptides that resolved at Mr 84,000, 47,000 and 25,000 which are consistent with immunoglobulin μ,γ, and light chains. Previous studies have shown that TABM (non-radioiodinated) detected in normal sera or purified from ascites fluid resolve as Mr 110,000 polypeptides which dissociate to the basic subunit of Mr 22,500 upon treatment with 5M guanidine [32, 33, 49]. Many investigators have also identified TABM after reduction as Mr 70,000 or
45,000 polypeptides [3, 7, 33, 44] which most likely represent multimers of the basic subunit.

*Scid* mice lack the ability to rearrange the V region in both the Igh and TCRβ genes, and thus fail to develop mature T or B lymphocytes [68, 69, 70]. Using this model we demonstrated the production of TABM, but not immunoglobulin, in thymocyte reconstituted, antigen stimulated, *scid* mice. This further demonstrated that TABM are indeed distinct from B cell-produced immunoglobulins. This model also demonstrated that B lymphocytes are not required for TABM production. In addition, TABM must require genetic mechanisms similar to the T cell receptor or immunoglobulin assembly to generate TABM because *scid* mice did not produce TABM prior to reconstitution.

TABM levels in normal serum are generally in the μg/ml range, although BALB/c mice have threefold higher amounts of endogenous TABM than CBA/J mice, which suggests that genetic background is an important factor in TABM levels [20]. Cone et al. [20] demonstrated a modest (1.5-2 fold) increase in TABM levels after immunization only, but observed a dramatic 300 fold increase in total serum TABM within 12 hr of a desensitizing dose of antigen given to primed mice. The authors noted an extremely short serum half-life and concluded that this was understandable based on the potent immunoregulatory properties of TABM. Our findings with respect to storage of TABM further substantiate this claim. When stored in the refrigerator for 10 days, the TABM were unable to bind to specific antigen. The sera that was aliquoted and placed in the freezer, however, maintained high binding ability. It is possible that an inactivator is present in
the sera which is unable to perform at extremely low temperatures. TABM are also extremely sticky proteins which tend to adhere to plastic or glass [49]. For this reason our sera was aliquoted and not refrozen after use. Possibly the TABM in the sera maintained in the refrigerator were also adhering to the plastic container and thus were unable to bind in ELISA.

The function of soluble TABM depends on their association with other non-antigen specific proteins. The TABM/accessory molecule complex has been found to be important in immunoregulation as helper factors [47, 56] or more commonly as suppressor factors [7, 11, 18, 22, 34]. They also have been demonstrated to transfer contact sensitivity in a manner similar to that of immediate type hypersensitivity [2, 16, 27, 28, 52]. In addition, TABM have been shown to regulate the IgE response through control of post translational glycosylation of IgE binding factors [71]. While the idiotypic determinants of TABM correlate with the antigen specificity of the TsF, the isotypic determinants correlate with their biologic activity. Different TABM subtypes have been identified based on their ability to react with distinct subsets of anti-TABM antisera (R.E.Cone, unpublished observation).

Although we have not employed functional assays, our data suggests that the TABM generated in this humoral immune response are the immunoregulatory type. The dose response data clearly shows that at high antigen doses (10 mg) TABM are produced at relatively high levels while immunoglobulin production is decreased dramatically. It is possible that the TABM are suppressing immunoglobulin production at these high antigen doses or they may be inducing suppression mediated by another TABM isotype. Additionally, the short half life for TABM activity when the serum is
maintained in the refrigerator suggests that an inactivator is present in the serum. This inactivator would be critical if the TABM were to have these potent immunoregulatory effects.

Antigen-specific immunoregulatory molecules have been shown to bear epitopes recognized by antibodies specific for T cell receptor alpha chain constant region [25, 41, 42, 61] although these molecules are not "conventional" T cell (membrane) receptor alpha chains. Also, TABM used to prepare anti-TABM antisera bear an epitope present in TcR VJβ [40]. Finally, TABM and immunoglobulin have been shown to cross react with antibodies produced against a synthetic peptide that corresponds to the J region of the TcR β gene. This data suggests that TABM, immunoglobulin and the TcR share serologic determinants. Our data with the scid mice suggest that TABM, like immunoglobulin and the TcR, require a rearranging V region gene. It is possible that TABM utilize the TcR α chain V region along with a new or modified constant region gene to form the molecule. Indeed, Zheng et al. [66] demonstrated that a TcR Vα gene is required to produce a functional antigen-specific immunoregulatory molecule. It may be possible, however, that TABM V genes may represent a third set of rearranging genes coding for antigen-specific molecules.
We have demonstrated a new humoral immune response by T lymphocytes. Upon stimulation with antigen, T cells are induced to produce soluble products which bind antigen (TABM). This response is specific for the inducing antigen and is stronger after challenge with antigen. The serum TABM do not maintain activity if stored at 4° for extended periods of time, but must be aliquoted and maintained at -20° for consistent results. The kinetics of TABM production differ temporally from immunoglobulin production and TABM production peaks at a higher dose of antigen than immunoglobulin. TABM were isolated by affinity chromatography and resolved at Mr 110,000, which is also unlike immunoglobulin. Finally, scid mice which have been reconstituted with thymocytes are able to produce TABM but not immunoglobulin, while unreconstituted scid mice do not produce TABM. These results indicate a new antigen specific humoral immune response by the T lymphocyte which may indicate a soluble extension of T cell function and potent immunoregulatory functions at the site of antigenic challenge in vivo.
REFERENCES


