JCI The Journal of Clinical Investigation

Molecular identification of T cells that respond in a primary bulk culture to a peptide derived from a platelet glycoprotein implicated in neonatal alloimmune thrombocytopenia.

K Maslanka, M Yassai, J Gorski

J Clin Invest. 1996;98(8):1802-1808. https://doi.org/10.1172/JCI118980.

Research Article

Neonatal alloimmune thrombocytopenia induced by the human platelet alloantigen 1a (HPA1a) is characterized by generation of alloantibodies by a mother who is homozygous for the HPA1b alloantigen and almost always HLA-DRB3*0101. The disease is viewed as B cell mediated but the linkage with HLA is indicative of a role for T cells. The HPA1a and HPA1b allotypes are defined, respectively, by Leu and Pro at amino acid 33 of the beta-chain of the platelet integrin GPIIbIIIa (alpha(IIb)beta3). Under the assumption that the same polymorphism may control both the B cell epitope and constitute the MHC-bound peptide, we restimulated PBMC from a woman with an affected child with a synthetic peptide from this polymorphic region. Molecular analysis of the responding T cell repertoire identified two T cells which predominated in cultures stimulated with the alloantigen peptide and which were absent in cultures with the autoantigen peptide. In spite of the use of different V families, sequence of the CDR3 region of the T cell receptor (TCR) beta-chain revealed the presence of a shared motif, L-P-S/T. Oligonucleotide probes specific for the CDR3 sequence indicated that these T cells were present in the PBMC at the highest levels immediately after delivery of the affected infant and their frequency dropped at later times.

Molecular Identification of T Cells That Respond in a Primary Bulk Culture to a Peptide Derived from a Platelet Glycoprotein Implicated in Neonatal Alloimmune Thrombocytopenia

Krystyna Maslanka, Maryam Yassai, and Jack Gorski
Immunogenetics Research Section, Blood Research Institute, The Blood Center of Southeastern Wisconsin, Milwaukee, Wisconsin 53201-2178

Abstract

Neonatal alloimmune thrombocytopenia induced by the human platelet alloantigen 1a (HPA1a) is characterized by generation of alloantibodies by a mother who is homozygous for the HPA1b alloantigen and almost always HLA-DRB3*0101. The disease is viewed as B cell mediated but the linkage with HLA is indicative of a role for T cells. The HPA1a and HPA1b allotypes are defined, respectively, by Leu and Pro at amino acid 33 of the β-chain of the platelet integrin GPIIbIIIa ($\alpha_{IIb}\beta_3$). Under the assumption that the same polymorphism may control both the B cell epitope and constitute the MHC-bound peptide, we restimulated PBMC from a woman with an affected child with a synthetic peptide from this polymorphic region. Molecular analysis of the responding T cell repertoire identified two T cells which predominated in cultures stimulated with the alloantigen peptide and which were absent in cultures with the autoantigen peptide. In spite of the use of different V families, sequence of the CDR3 region of the T cell receptor (TCR) β-chain revealed the presence of a shared motif, L-P-S/T. Oligonucleotide probes specific for the CDR3 sequence indicated that these T cells were present in the PBMC at the highest levels immediately after delivery of the affected infant and their frequency dropped at later times. (*J. Clin. Invest.* 1996. 98:1802–1808.) Key words: alloantigen • platelet • T cell antigen receptor, beta chain • gene rearrangement • complementarity determining region

Introduction

Alloimmunity is generally considered as an important component in transplantation and the MHC is one of the most potent alloantigen systems. However, alloimmunity can also be a complication of pregnancy or transfusion. While the MHC again plays an important role, other antigen systems contribute substantially. Such non-MHC antigen systems include the polymorphic proteins expressed on other circulating cells. Among these, the platelet alloantigen system associated with surface integrin GPIIb/IIIa ($\alpha_{IIb}\beta_3$) plays an important role in pregnancy and transfusion related alloimmunity (1, 2).

A characteristic of these alloimmune responses is the presence of circulating antibodies specific for the alloantigen. These are thought to mediate the destruction of the cells expressing the alloantigen. One particular alloimmune response is interesting in its HLA restriction. The GPIIb/IIIa alloantigens HPA1a and HPA1b are defined by antibodies which are sensitive to the polymorphism at position 33 of the GPIIIa chain (3). Mutagenesis experiments have shown that this polymorphism is sufficient for inducing alloantibody recognition (4). The generation of a response to HPA1a ($GPIIIa_{Leu33}$) requires that the responder be HPA1b ($GPIIIa_{Pro33}$) homozygous. The resulting platelet destruction gives rise to post-transfusion thrombocytopenia purpura (PTP) or neonatal alloimmune thrombocytopenia (NAIT).[1] Interestingly, responders are almost always HLA DRB3*0101 (5) or DQB1*02 (6). For NAIT, the relative risk for DRB3*0101 has been calculated as 141, which is of the same order as for one of the best examples of an HLA-linked autoimmune disease, ankylosing spondylitis and HLA B27. Also of interest is that the reverse response (anti-HPA1b) is much less frequent and does not show an HLA linkage (7).

The contribution of B cells to this response has been studied by identification of factors involved in generating the epitopes on GPIIIa recognized by these cells. Not only has the controlling polymorphism been identified for this response, a number of experiments have led to the concept that the B cell epitope is complex (8) and may involve contributions from various parts of the GPIIIa chain (9, 10).

While the B cell contribution has been the subject of much study, the contribution of T cells has been generally ignored. The high degree of HLA restriction in this response as well as the fact that the antibodies are IgG indicate that the B cells require T cell help. In this paper, we worked under the hypothesis that the polymorphic region controlling the antibody epitope could also function as the antigenic peptide responsible for this stimulation, a hypothesis which had been supported by preliminary experiments (11). We used a combination of approaches to identify two likely T cell clonotypes involved in this response using the TCR β-chain as a clonotypic marker. Clonotype-specific probes were used to investigate the levels of these T cells at various times after pregnancy.

Methods

Cells. The cells of a healthy Caucasian woman who had delivered two babies with NAIT were investigated. The woman was typed as

Address correspondence to Jack Gorski, Blood Research Institute, Blood Center of S.E. Wisconsin, P.O. Box 2178, Milwaukee, WI 53201-2178. Phone: 414-937-6367; FAX: 414-937-6824; E-mail: jack@bcsew.edu. Krystyna Maslanka's current address is Institute of Haematology and Blood Transfusion, Department of Serology, ul.Chocimska 4, 00-957 Warsaw, Poland.

Received for publication 29 January 1996 and accepted in revised form 26 July 1996.

J. Clin. Invest.

0021-9738/96/10/1802/07 $2.00
Volume 98, Number 8, October 1996, 1802–1808

1. *Abbreviations used in this paper:* NAIT, neonatal alloimmune thrombocytopenia; TCR, T cell receptor.

HPA-1b and HLA DRB1*02, DRB1*03, DRB3*0101. Circulating anti-HPA1a antibodies were found after both the first and second pregnancy using antigen capture ELISA. Blood samples were obtained under an Institutional Review Board (IRB) approved protocol.

PBMC obtained from whole citrate blood were isolated by Ficoll-Hypaque (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) gradient and used throughout this procedure. PBMC were collected at different times after delivery of the second child (6, 18, 24, 30, and 42 mo, respectively).

Platelet membranes were prepared (12) from the platelet-rich plasma fractions collected from HPA1a and HPA1b homozygous individuals. The platelet donors were unrelated to the subject of the study. All blood samples were obtained with informed consent and under IRB approval.

Culture conditions. Culture was performed using PBMC isolated 30 mo after delivery, in RPMI-1640 culture medium (Whittaker, Rockville, MD) supplemented with 10% heat inactivated AB pool human serum, 1 mM L-glutamine, 1 mM sodium pyruvate, 10 IU/ml heparin, 4 μg/ml gentamicin, 100 μg/ml streptomycin, 100 μg/ml penicillin and 25 mM HEPES buffer, in 6-well flat bottom plates (Costar Corp., Cambridge, MA) which were kept in incubator at 37°C in a humidified 5% CO_2 air atmosphere.

PBMC (10^6 cells/ml per well) were initially incubated with different concentrations (50 μM, 25 μM, 12.5 μM, or 2 μM) of synthetic peptides GPIIIa $_{26–38leu33}$ or GPIIIa $_{26–38pro33}$ (as negative control). After 7 d, the cultures were divided in two parts. To both parts, fresh irradiated autologous PBMC (10^6 cells/ml) and the same appropriate concentration of peptide were added. To one part 50 IU/ml recombinant human IL-2 (Genzyme Corp., Cambridge, MA) was added and culture continued for 7 more days. After 14 d, cells were harvested, washed in PBS, and used for RNA preparation.

Peptide synthesis. The peptide GPIIIa$_{26–38leu33}$ and GPIIIa$_{26–38pro33}$ were synthesized on Pepsyn KA resin (Millipore Corp., Bedford, MA) using a 9050 Pepsynthesizer (Millipore Corp.) according to the manufacture-specified protocols. The peptides were purified by reverse phase HPLC (Beckman Instruments, San Ramon, CA) to > 90% purity using a C18 column (Vydac, Hesperia, CA). Fast atom bombardment mass spectra were obtained for the purified peptide and verified the expected weight. The sequence of the two peptides is as shown: GPIIIa$_{26–38leu33}$, NH_2-CSDEALPLGSPRC-COOH. GPIIIa$_{26–38pro33}$, NH_2-CSDEALPPGSPRC-COOH. The allelic differences are underlined.

The purified peptides were cyclized by forming disulfide bridges through air oxidation in 0.1 ammonium acetate buffer (pH 6.0) at a concentration of 0.1 mg/ml. The cyclized product was then repurified by (RP)HPLC. The pre- and post-oxidative products were then assayed using quantitative Ellman's test and FAB mass spectrophotometry.

RNA and cDNA preparation. RNA was prepared according to Chirgwin et al. (13) by homogenizing the cells in guanidinium isothiocyanate and pelleting in CsCl with a final ethanol precipitation. 1 μg of total RNA was converted into cDNA using oligo (dT) as primer for reverse transcription as described earlier (14).

PCR amplification. PCR amplification of cDNA was performed as previously described (15). Briefly, the cDNA volume corresponding to 20 ng of total RNA was amplified in 30 cycles with 24 TCR BV family-specific primers (BV) and a β-chain constant primer (BC). The BC primer was labeled at the 5′ end with 5′6 carboxyfluorescein (Pharmacia LKB Biotechnology Inc.). To avoid 24 different amplification of cDNA with all TCR BV family specific primers, 11 TCR BV primer pairs were used in multiplex PCR. The two primers also act as controls for each other in the PCR. Only for BV6 family was simplex PCR used. The sequences of the constant primer, the 24 TCR BV family-specific primers and their pairing for multiplex PCR, were published earlier (15).

TCR spectratyping. This technique has been previously described in detail (15). An equivalent volume of PCR-labeled product was mixed with formamide dye loading buffer, heated at 94°C for 2 min, and applied to a prewarmed 5% acrylamide-urea sequencing gel. Gels were run 100 min to resolve the region corresponding to 120–220 bp. The labeled PCR product was analyzed by Molecular Dynamics FluorImager 575 (Sunnyvale, CA).

Subcloning and DNA sequencing. The TCR β-chain cDNA corresponding to specific spectratype bands were subcloned by performing a separate PCR using subcloning primers. These contained an additional PstI restriction site on the BV primers or a BamHI site on the C region primer. The primer sequences are: BV5.3, CTCAGGTCTGCAGTTCCCTAACTATAGCTCTGAGC; BV11, GAGTCTGCAGTCTCCAGAATAAGGACGGAGC; and BC, GAGCCATCAGAAGCAGAGATCTGGATCCCCCA. The C region primer is synthesized as inverse complement of the sequence shown here.

PCR products were purified with phenol/chloroform extraction followed by ethanol precipitation. After resuspension in a small volume of water, the DNA was digested with the appropriate enzymes and ligated into digested pGEM 3zf(f) plasmid vector (Promega Corp., Madison, WI). The sequence analysis was performed using Taq Dye Deoxy-Terminator Cycle Sequencing Kit (Applied Biosystems Inc., Foster City, CA).

Colony and electroblot hybridization. Probes specific for the CDR3 of the TCR (clonotype-specific probes) were designed on the basis of the DNA sequences generated (Table I). The probe sequence for the BV4 clonotype is GAGGGGGGGACCTCCTACG. Optimal hybridization and washing conditions were determined using plasmid cloned DNA. The probes were labeled using $^{32}P\gamma$ATP and polynucleotide kinase and used for colony hybridization as described (16). Spectratypes were electroblotted from acrylamide gels onto nylon membranes (0.22 mm; Magna Graph, MSI, Westboro, MA). The DNA was crosslinked for approximately 30 s UV using a Stratalinker UV 1800 (Stratagene Inc., La Jolla, CA), baked at 80°C for 60 min, and hybridized in 50°C with ^{32}P-labeled clonotype-specific probes. Hybridization was conducted in hybridization buffer (10 × Denhard solution, 5 × SSC, 7% SDS, 20 mM Na_2HPO_4 and 100 μg/ml denatured herring sperm DNA) at 50°C overnight. The membranes were washed at 50°C twice in 10 × Denhard solution, 5% SDS, 70 μM phosphate buffer, pH 7.0 and twice in 1 × SSC, 1% SDS and subjected to autoradiography.

After clonotype-specific probe hybridization, the radioactive probe was stripped off (100°C, H_2O) and membranes were rehybridized with BC-specific oligonucleotide probe in the same condition as described above. An AMBIS Scanner (Ambis, San Diego, CA) or a Molecular Dynamics PhosphoImager was used to directly count the total radioactivity in each band.

Calculation for relative frequency of TCR clones. The frequency of T cell clonotypes was calculated using the cpm/band after hybridization with clonotype-specific probes and rehybridization with BC-specific probe.
It is calculated as:

$$\frac{\text{cpm/band clonotype-specific probe}}{\text{cpm/band BC-specific probe}} \times \text{correction factor.}$$

The correction factor is derived from hybridization of DNA from plasmid clone as:

$$\frac{\text{cpm/band BC-specific probe}}{\text{cpm/band clonotype-specific probe}}$$

Results

Identification of responding T cells. The contribution of T cells to NAIT was studied by identifying T cells that expand or survive in primary bulk cultures. This form of culture was chosen as it represents a minimum deviation from the peripheral environment. Culture was for 1 wk without growth factors. After restimulation, a second week of culture was performed in the presence or absence of recombinant IL-2. Control cultures without peptide or with an autologous peptide were per-

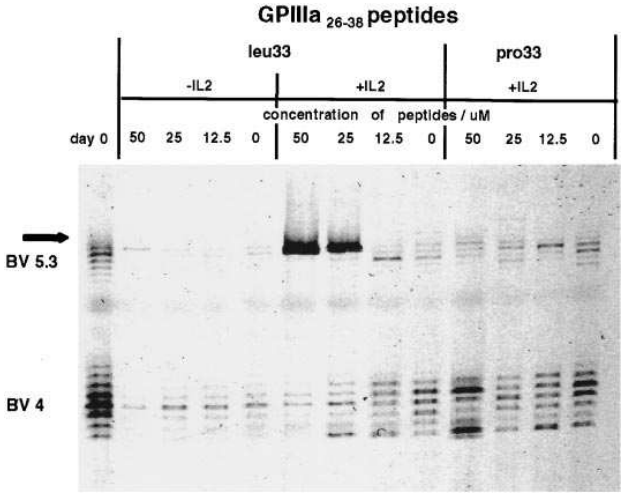

Figure 1. BV5.3 and BV4 spectratypes of PBMC after 14 d of culture with allogeneic peptide GPIIIa$_{26–38leu33}$, left and center and autologous peptide GPIIIa$_{26–38pro33}$. Data in the left lanes are from cultures without IL-2 stimulation. Concentration of peptide used is shown above each lane. The *lane* labeled 0 day is the starting PBMC repertoire. The bands corresponding to the BV5.3 and BV4 spectratypes are identified on the left. The arrow points to the specific band enhancement.

formed in the same manner. Varying concentrations of peptide were used.

PBMC from a woman who had two affected pregnancies were used. These studies were performed on PBMC obtained 30 mo after the second pregnancy. At this time there was still a circulating titer of anti-HPA1a antibodies as measured by modified antigen capture ELISA.

T cells specific to cultures stimulated by the alloantigenic peptide were identified by their CDR3 length, an assay we refer to as a spectratype. A typical PBMC spectratype shows a Poisson distribution of 6–14 bands with a 3-bp spacing corresponding to T cells with expressing TCR mRNA of different CDR3 lengths. We use a bifamily PCR approach with two BV families coamplified in the same PCR. This reduces the complexity of the analysis and the two families act as controls for each other (15).

An example of the results obtained is shown in Fig. 1. The lane labeled 0 day shows the PBMC spectratype for the BV5.3 and BV4 families with 7 and 9 bands, respectively. After 2 wk of culture, the IL-2+ culture shows a very intense BV5.3 band in the presence of the GPIIIa$_{26–38leu33}$ allopeptide, signifying T cell expansion. This band is not seen in the absence of peptide (lane where concentration = 0) nor in cultures with the pro33 peptide. This signifies allopeptide-specific expansion. At 50-uM peptide the same band is the most predominant band and can be observed even in the absence of IL-2.

For the BV4 family (lower spectratype) there is no specific band increase in the cultures with peptide. The presence of IL-2 increases the intensity of some bands, but the same increase is seen in the absence of peptide. This type of negative result was seen for many other V families. Other negative patterns included no visible signal, or sporadic increases in band intensities, usually in the presence of IL-2.

Only the BV11 showed another example of specific response and the results are shown in Fig. 2. A very intense band is seen in the presence of the leu33 allopeptide. This expansion is seen even in the culture without IL-2. In the presence of IL-2, the increase in band intensity is seen even in the 12.5-μM culture, thus the peptide concentration appears to be saturating under these conditions. While the intensity is lower in the 25 μM + IL-2 culture, it still represents one of the two predominant species in the culture. Thus, these three cultures could be viewed as triplicates with excellent expansion occurring in two of the three cultures with the third culture showing adequate expansion. In the absence of IL-2, there is a definite dose dependence on the intensity of the band. As can be observed at 0 day, BV19 does not show a 3-bp pattern. This is due to defective expression and thymic selection (17).

Therefore, two likely examples of specific T cell expansion were identified after this analysis.

Sequence and clonotype analysis. An intense spectratype band could represent one or a multitude of unique clonotypes. Therefore, the sequence complexity of each band was examined. The PCR products from cultures in which one intense band was present was used for bacterial cloning and sequence analysis. For the first round of sequencing four and five bacterial colonies containing BV5.3 and BV11 inserts, respectively, were chosen for sequencing. For each BV family, all the colonies gave identical sequences (Table I). The two TCR thus identified used different J regions. A striking observation is that while the DNA sequences are different, there is a conservation of amino acid sequence, indicating the use of a shared recognition motif in the CDR3 of the β-chain. The motif consists of two identical residues, L and P, followed by S or T. The fourth position represents the use of an amino acid with a very small side chain.

Clonotype-specific oligonucleotide probes (bold in Table I) were generated on the basis of these sequences and used for hybridization of the remaining bacterial colonies. Of the colonies with BV5.3 inserts, 76% hybridized with the probe. The number was even higher for the BV11 hybridization, 94%. Thus, on the basis of colony counting, the bands appear to be predominantly represented by one sequence.

An independent test of the actual BV5.3 and BV11 PCR products was performed to determine the contribution of these clonotypes to the signals observed in the spectratype analyses of the cultures. New spectratypes were generated,

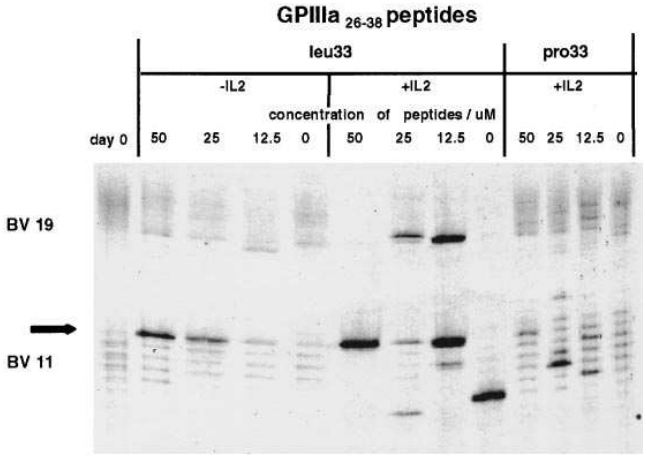

Figure 2. BV19 and BV11 spectratype analysis of 14-d cultures of PBMC. Labeling is as described for Fig. 1.

Table I. Sequences of CDR3 Region Specific for T Cell Clones BV5.3 and BV11

BV 5.3 clone (four different clones sequenced)
BV5.3 D1 BJ 2.3
TGT GCC AGC A**GC TTG CCG ACA GGG ATC**
GGC ACA GAT ACG CAG
Cys Ala Ser Ser Leu Pro Thr Gly Ile Gly Thr Asp Thr Gln

BV 11 clone (five clones)
BV 11 D2 BJ 2.2
TGT GCC AGC A**GT CTT CCT AGC GCC CAA**
AAC ACC GGG GAG
Cys Ala Ser Ser Leu Pro Ser Ala Gln Asn Thr Gly Glu

The sequences used for clonotype-specific probes are in bold.

electroblotted to nylon, and hybridized with the clonotype-specific probe. After phosphorimaging, the membranes were stripped and hybridized with a C region probe. This analysis is shown in Fig. 3 for BV5.3 and Fig. 4 for BV11. It can be seen that these clonotypes are present in all the cultures in which the original band intensity increase had led us to hypothesize a specific expansion.

The same approach was used to independently estimate the contribution of the clonotype to the total signal of the band. The cDNA from culture after 50 μM peptide stimulation +IL-2 was used for this analysis. To insure that the PCR data could be used for quantitation, a dilution series of cDNA was used for the PCR step and reactions where band intensity was a linear function of amount amplified were analyzed. To correct for differences in efficiencies between the clonotype and C region probes, a dilution series of plasmid containing the cDNA insert was also hybridized. As the target ratio of clonotype to C region is 1:1 on the plasmid clone, differences in hybridization intensity represent differences in probe efficiency (labeling or hybridization) and must be corrected for. The results of these measurements and calculations indicated that BV5.3 clonotype represented 86% of the total band intensity. This is in keeping with the 76% obtained from colony counting. The same analysis gave 110% for BV11 which again is close to the 94% obtained by colony counting. Therefore, the response appears to be monoclonal for both these BV families.

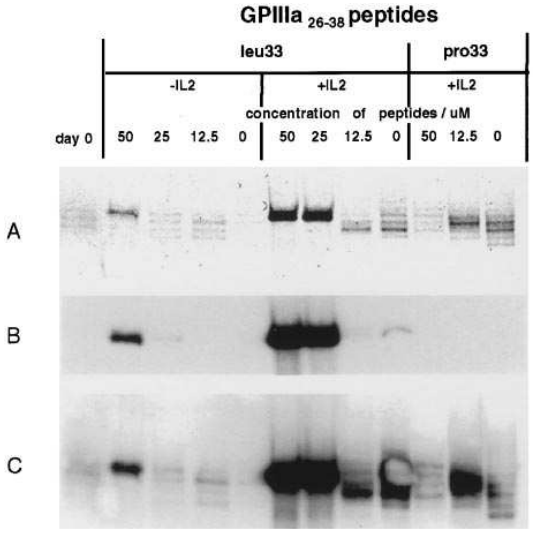

Figure 3. Hybridization analysis of the BV5.3 clonotype. (*A*) BV5.3 spectratype generated as in Fig. 1. (*B*) Hybridization of DNA shown in *A* with BV5.3 clonotype-specific probe after electroblotting onto a nylon membrane. (*C*) Rehybridization of the membrane shown in *B* with BC-specific probe.

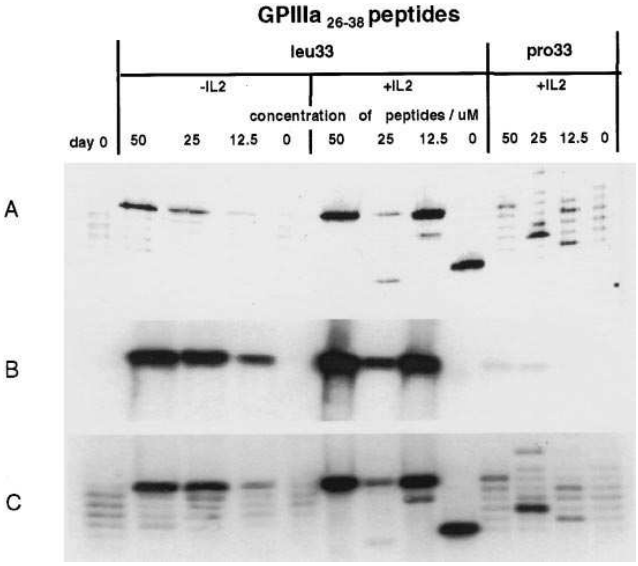

Figure 4. Hybridization analysis of the BV11 clonotype. (*A*) BV11 spectratype of cultures as described in Fig. 2. (*B*) Hybridization BV11 clonotype-specific probe. (*C*) Rehybridization of membrane shown in *B* with BC-specific probe.

Clonotype frequencies in peripheral blood as a function of time after pregnancy. If the two clonotypes identified by their ability to respond in vitro are really involved as part of NAIT, then their frequency should be higher in unstimulated PBMC obtained closer to the time of delivery, and thus closer to the

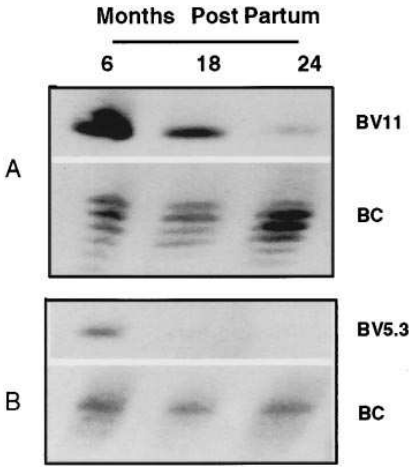

Figure 5. Measurement of clonotype-specific signal from spectratypes generated from PBMC obtained at different times after delivery. (*A*) BV11 spectratype. The top shows hybridization with the clonotype-specific probe. The bottom shows rehybridization with BC-specific probe. (*B*) BV5.3 spectratype. The panels are as described for *A*.

Table II. Direct Measurement of Clonotype-specific Probe Hybridization to PCR Products Generated from cDNA Isolated at Different Time Post-partum

^{32}P-labeled probes	Months post-partum		
cpm/band	6	18	24
BV11	24.8	7.3	3.4
BC	63.9	64.3	109.2
Percent BV11/BC	38.8	11.3	3.1
BV4	132.2	121.6	73.4
BC	1646.3	2218.8	785.2
Percent BV4/BC	8.0	5.5	9.3

time of the last stimulation in situ. PBMC samples were available from a number of time points after delivery of the last affected child. BV5.3 and BV11 spectratypes were generated and used for clonotype hybridization. The autoradiograms of both analyses are shown in Fig. 5. The signal for the clonotype-specific probe is shown along with the signal for the same electroblot after rehybridization with a C region probe. It is clear that the clonotype signal at the earliest time point (6 mo after delivery) is the most intense relative to the C region signal. For the BV11 clonotype, the signals were sufficient for quantitation directly from the filter (Table II). The counts per band are shown for the BV11- and BC-specific probes. The clonotype frequency is calculated as the percent ratio of BV11/BC cpm/band. The clonotype frequency is highest right after delivery and decreases at later times. It should be pointed out that at 30 mo after delivery the signal in the unstimulated PBMC is almost undetectable (0 day; Figs. 3 and 4).

A nonresponsive clonotype does not show a relation between frequency in peripheral blood as a function of time after pregnancy. To rule out any possible large scale turnover of T cell clonotypes as a general characteristic of the period immediately after pregnancy, a random clonotype was assayed in the same manner as described above. This BV4 clonotype was identified from an anchor PCR-amplified TCR cDNA library obtained from blood sampled at time of delivery (11). This BV4 clonotype was a high frequency component at that time (11) and thus would make an excellent candidate for measuring nonspecific repertoire turnover. The same cultures were analyzed for the presence of this clonotype and the 14-d results with IL-2 are shown in Fig. 6 (*left*). The clonotype did not respond in cultures with the leu_{33} peptide and IL-2, nor did it show a decrease in frequency in PBMC obtained at later times after pregnancy (Fig. 6, *right*). The calculated hybridization intensities of the clonotype-specific probe for the same three time points as were calculated for the BV11 clonotype are shown in Table II.

The peptide-specific T cells can respond to allogeneic platelet membranes. The significance of these observations could be enhanced by showing that the T cells can respond to allogeneic but not autologous platelet membranes. A sonicated platelet membrane preparation derived from either an HPA1b or from an HPA1a individual was used to stimulate PBMC (18-mo sample). The culture was without added lymphokines and analyzed after 4 and 7 d. Fig. 7 shows the results of hybridization of a VB11 spectratype with the BV11 clonotype-specific probe and subsequent rehybridization with a C region probe. It is evident that the allogeneic HPA1a platelet membranes were stimulatory whereas the HPA1b platelet membranes were not. A response could be observed by day 4 indicating a rapid kinetic.

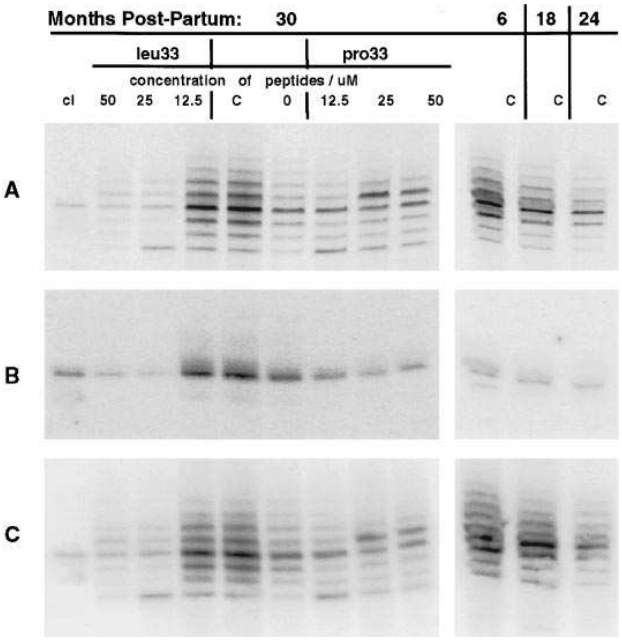

Figure 6. Analysis of a random clonotype present at high frequency during delivery. (*A*) The BV4 spectratypes for the in vitro cultures (*left*) and from PBMC at various times after delivery (*right*). In the left panel *CL* refers to the BV cDNA clone and *0* to culture without peptide. In both panels *C* refers to uncultured PBMC. Culture conditions are specified above each lane. (*B*) The gel in *A* was electroblotted and hybridized with the clonotype-specific probe. (*C*) The membrane in *B* was stripped and rehybridized with a C-region probe.

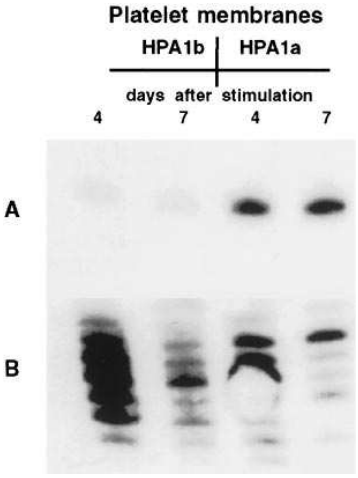

Figure 7. Clonotype hybridization after culture with HPA1a and HPA1b platelet membranes. The HPA type of the stimulating platelets is described above each pair of lanes and the time of analysis specified above each lane. (*A*) Hybridization with the BV11 clonotype-specific probe. (*B*) Rehybridization of the membrane with a C-region probe.

Discussion

We present here an analysis of a recall response to a non-HLA platelet alloantigen which can have a clinical manifestation during pregnancy. This analysis is performed using defined peptides for T cell restimulation and the responding repertoire is analyzed using molecular biological techniques. The alloantigen, HPA-1a has been extensively studied from the B cell perspective (9, 18), but the contribution of the T cell response has been relatively ignored. A T cell component is strongly suspected because of the class II HLA linkage (5). We hypothesized that a woman who had generated alloantibodies, gave birth to an affected infant, and whose circulating antibody titers were still measurable would be an excellent source of memory T cells for a recall response. We also hypothesized that at least a part of the T cell response may be due to recognition of the same polymorphism that is responsible for controlling the B cell epitope. Peptides from this region were synthesized and used for the restimulation assays. The HLA-DR binding properties of these peptides is the subject of a separate study (S. Wu, K. Maslanka, and J. Gorski, manuscript in preparation).

The CDR3 length heterogeneity of the BV chain was used to measure in vitro repertoire complexity for each BV family. The BV chain can be used as a clonotypic marker to follow expansion of a particular T cell lineage as generation of one functional β-chain stops further BV recombination (19). Thus, there is usually only one BV gene expressed per cell (20–22). CDR3 length analysis has been used to analyze the response to pigeon cytochrome *c* and this analysis was aided by a final in vitro expansion (23). We have also applied this form of analysis to short term cultures stimulated with matrix and nucleoprotein peptide antigens from influenza virus (M. Yassai and J. Gorski, manuscript in preparation).

The short term cultures used for restimulation were performed under relatively simple conditions. A very restricted T cell response was observed with the same bands appearing in cultures performed at different times. Detailed analysis showed that there were two predominant clonotypes involved. They shared a recognizable CDR3 motif but used two different BV families. The presence of a CDR3 motif is taken to indicate TCR selection for antigen recognition (24). There are numerous examples where a class II MHC restricted response shows this characteristic for the BV or AV chains (25–32). Finding a CDR3 motif associated with two different BV genes in the same animal or individual is much less common (33). However, in vitro mutagenesis has shown that for moth cytochrome *c* peptide, the transfer of a CDR3 onto an unrelated BV gene reconstituted partial recognition (34). The presence of the BV CDR3 motif supports the relevance of the clonotype expansions. Both of the clonotypes were specific for the HPA-1a polymorphism as they would not be stimulated by a peptide with proline at position 33, as this represents a self peptide. These BV clonotypes identify a minimum of two responding T cell lineages.

The biologic relevance of these clonotypes was confirmed by the observation that these clonotypes constitute a more substantial percentage of the circulating repertoire close to time of delivery, when stimulation of maternal T cells by fetal platelet is expected to be higher. The relative frequency decreased post-partum. It was difficult to show leu_{33} peptide-specific expansion of these clonotypes in culture using PBMC from a later (42 mo) time point and expansion was partially masked in the 16-mo time point by the high starting level. Thus, there can be a window during which time the specific recall response may be most easily studied. A control clonotype, chosen at random from high frequency clonotypes present at time of delivery, did not show a leu_{33} peptide-specific response nor a decrease with time post-partum. The observation that allogeneic platelet membranes were very efficient at stimulating the BV11 clonotype also indicates the relevance of these findings.

Thus, these T cells are very likely candidates for providing the HLA-DR restricted help for generation of the antiplatelet alloantibodies associated with NAIT. While these results implicate these T cell clonotypes in providing help for B cells, and represent the strongest current evidence for T cell involvement in antibody production to alloantigens, this is still indirect. Further experiments directly linking the presence of these clonotypes with ability to produce alloantibody in vitro will be necessary. It will also be interesting to link the nature of the antibody produced (8, 9, 35) with the nature of the T cell response. These correlations await further understanding of the variables involved in initiation of T cell responses. The penetrance of NAIT in HPA-1b, DRB3*0101 mothers with HPA-1a infants is low (36) arguing for the presence of additional factors.

The experiments presented here also show that peptides that contain polymorphism that control B cell epitopes can also stimulate T cells. In the case of the anti-HPA1 response studied here, this is not meant to imply that other peptides from either the β_3 or α_{IIb} chains may not also participate in stimulating maternal T cells. Both these subunits are polymorphic (reviewed in reference 10) and any additional differences between mother and fetus may increase the chance of induction of an antibody response. However, inclusion of an additional polymorphism at position 40 of the β_3 chain (37) did not influence the response controlled by leu33 when a longer peptide was used (unpublished observations) with the PBMC studied here. It is also possible that in some instances the polymorphism controlling the B cell epitope and the T cell epitope may be derived from separate peptides, analogous to a hapten-carrier response. While this was not the case here, studies of additional HPA1 cases or of other platelet alloantigens may reveal such occasions.

The molecular analysis of NAIT presented here can be used as a paradigm for other alloantibody mediated responses. It is noteworthy that in spite of the many years that anti-HLA alloantisera have been collected from multiparous women and used for HLA typing, there is a profound ignorance of the details as to how these alloantibodies are generated. For alloantibody responses with clinical repercussions, the identification of T cells involved in alloantigen responses, especially ones sharing CDR3 motifs, may provide novel avenues of intervention.

Acknowledgments

We thank Drs. Janice McFarland and Joan Gill for help in obtaining PBMC, M. Trudy Holyst for GPIIIa peptide synthesis, Charles Brierre for synthesizing oligonucleotides, The Platelet Antibody Lab and the DNA Diagnostic Lab of the Blood Center of Southeastern Wisconsin for HPA and HLA-DR typing, respectively, Dr. Yuri Naumov for teaching the clonotyping procedure, Drs. Debra Newton-Nash and

Raffaele DePalma for help and discussion relating to cell culture, and Dr. Peter Newman for general discussions about alloimmune thrombocytopenia.

This work was supported by National Institutes of Health grants PO1-HL44612 and RO1-HL49747 to J. Gorski and general Clinical Research Center Grant RR-00058-32 to the Medical College of Wisconsin.

References

1. Newman, P.J., J.G. McFarland, and R.H. Aster. 1994. Alloimmune thrombocytopenia. *In* Thrombosis and Hemorrhage. Loscalzo, J., and A.I. Schafer, editors. Blackwell Scientific Publications, Boston. 529–543.

2. Panzer, S., L. Auerbach, E. Cechova, G. Fischer, A. Holensteiner, E.M. Kittl, W.R. Mayr, M. Putz, P. Wagenbichler, and S. Walchshofer. 1995. Maternal alloimmunization against fetal platelet antigens: a prospective study. *Br. J. Immunol.* 90:655–660.

3. Newman, P.J., R.S. Derbes, and R.H. Aster. 1989. The human platelet alloantigens, Pl^{A1} and Pl^{A2}, are associated with a $leucine_{33}/proline_{33}$ amino acid polymorphism in membrane glycoprotein IIIa, and are distinguishable by DNA typing. *J. Clin. Invest.* 83:1778–1781.

4. Goldberger, A., M. Kolodziej, M. Poncz, J.S. Bennet, and P.J. Newman. 1991. Effect of single amino acid substitutions on the formation of the Pl^{A} and Bak alloantigenic epitopes. *Blood.* 78:681–687.

5. Valentin, N., A. Vergracht, J.D. Bignon, C.L. Cheneau, D. Blanchard, C. Kaplan, M.F. Reznikoff-Etievant, and J.Y. Muller. 1990. HLA-DRw52a is involved in alloimmunization against Pl^{A1} antigen. *Hum. Immunol.* 27:73–79.

6. L'Abbe, D., L. Tremblay, M. Filion, L. Busque, M. Goldman, F. Decary, and P. Chartrand. 1992. Alloimmunization to platelet antigen HPA-1a (PlA1) is strongly associated with both HLA-DRB3*0101 and HLA-DQB1*0201. *Hum. Immunol.* 34:107–114.

7. Kuijpers, R.W.A.M., A.E.G.K. Von dem Borne, V. Kiefel, C.M. Eckhardt, A.H. Waters, B. Zupanska, D. Barz, E. Taaning, A. Termijtelen, and W.H. Ouwerhand. 1992. $Leucine^{33}$-$proline^{33}$ substitution in human platelet glycoprotein IIIa determines HLA-DRw52a (Dw24) association of the immune response against HPA-1a (Zw^{a}/Pl^{A1}) and HPA-1b (Zw^{b}/Pl^{A2}). *Hum. Immunol.* 34:253–256.

8. Valentin, N., G.P. Visentin, and P.J. Newman. 1995. Involvement of the cysteine-rich domain of glycoprotein IIIa in the expression of the human platelet alloantigen, PlA1: evidence for heterogeneity in the humoral response. *Blood.* 85:3028–3033.

9. Honda, S., Y. Honda, B. Bauer, C. Ruan, and T.J. Kunicki. 1995. The impact of three-dimensional structure on the expression of Pl^{A} alloantigens on human integrin b_3. *Blood.* 86:234–242.

10. Newman, P.J., and N. Valentin. 1995. Human platelet alloantigens: recent findings, new perspectives. *Thromb. Haemostasis.* 74:234–239.

11. Yassai, M., J.G. McFarland, D. Newton-Nash, P.J. Newman, D.D. Eckels, and J. Gorski. 1992. T cell receptor and alloimmune thrombocytopenias: a model for autoimmune diseases? *Ann. Med. Interne.* 143:365–370.

12. Newman, P.J., R.A. Kahn, and A. Hines. 1981. Detection and characterization of monoclonal antibodies to platelet memebranes. *J. Cell Biol.* 90:249–253.

13. Chirgwin, J.M., A.E. Przybyla, R.J. McDonald, and W.J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry.* 18:5294–5299.

14. Gorski, J., M. Yassai, X. Zhu, B. Kissella, C. Keever, and N. Flomenberg. 1994. Circulating T cell repertoire complexity in normal individuals and bone marrow recipients analyzed by CDR3 size spectratyping. Correlation with immune status. *J. Immunol.* 152:5109–5119.

15. Maslanka, K., T. Piatek, J. Gorski, M. Yassai, and J. Gorski. 1995. Molecular analysis of T cell repertoires. Spectratypes generated by multiplex polymerase chain reaction and evaluated by radioactivity or fluorescence. *Hum. Immunol.* 44:28–34.

16. Grunstein, M., and D. Hogness. 1975. Colony hybridization: a method for the isolating of cloned DNA's that contain a specific gene. *Proc. Natl. Acad. Sci. USA.* 72:3961–3973.

17. Currier, I., M. Yassai, M.A. Robinson, and J. Gorski. 1995. Molecular defects in TCRBV genes preclude thymic selection and limit the expressed TCR repertoire. *J. Immunol.* 157:170–175.

18. Honda, S., Y. Tomiyama, A.J. Pelletier, D. Annis, Y. Honda, R. Orchekowski, Z. Ruggeri, and T.J. Kunicki. 1995. Topography of ligand-induced binding sites, including a novel cation-sensitive epitope (AP5) at the amino terminus, of the human integrin b_3 subunit. *J. Biol. Chem.* 270:11947–11954.

19. Uematsu, Y., S. Ryser, Z. Dembic, P. Borgulya, P. Krimpenfort, A. Berns, H. von Boehmer, and M. Steinmetz. 1988. In transgenic mice the introduced functional T cell receptor beta gene prevents expression of endogenous beta genes. *Cell.* 52:831–841.

20. Matis, L.A., A. Ezquerra, and J.E. Coligan. 1988. Expression of two distinct T cell receptor alpha/beta heterodimers by an antigen-specific T cell clone. *J. Exp. Med.* 168:2379–2384.

21. Padovan, E., C. Giachino, M.Cella, S. Valitutti, O. Acuto, and A. Lazavecchia. 1995. Normal T lymphocyte can express two different T cell receptor b chains: implications for the mechanism of allelic exclusion. *J. Exp. Med.* 181: 1587–1591.

22. Davodeau, F., M.A. Peyrat, F. Romagne, A. Necker, M.M. Hallet, H. Vie, and M. Bonneville. 1995. Dual T cell receptor b chain expression on human T lymphocytes. *J. Exp. Med.* 181:1391–1398.

23. Cochet, M., C. Pannetier, A. Regnault, S. Darche, C. Leclerc, and P. Kourilsky. 1992. Molecular detection and in vivo analysis of the specific T cell response to a protein antigen. *Eur. J. Immunol.* 22:2639–2647.

24. Hedrick, S.M., I. Engel, D.L. McElligot, P.J. Fink, M.L. Hsu, D. Hansburg, and L.A. Matis. 1988. Selection of amino acid sequences in the beta chain of the T cell antigen receptor. *Science (Wash. DC).* 239:1541–1544.

25. Fink, P.J., L.A. Matis, D.L. McElligot, M. Bookman, and S.M. Hedrick. 1986. Correlations between T cell specificity and the structure of the antigen receptor. *Nature (Lond.).* 321:219–226.

26. Winoto, A., J.L. Urban, N.C. Lan, J. Goverman, L. Hood, and D. Hansburg. 1986. Predominant use of a V alpha gene segment in mouse T cell receptors for cytochrome c. *Nature (Lond.).* 324:679–682.

27. Acha-Orbea, H., D.J. Mitchell, L. Timmerman, D.C. Wraith, G.S. Tausch, M.K. Waldor, S.S. Zamvil, H.O. McDevitt, and L. Steinman. 1988. Limited heterogeneity of T cell receptors from lymphocytes mediating autoimmune encephalomyelitis allows specific immune intervention. *Cell.* 54:263–273.

28. Urban, J.L., V. Kumar, D.H. Kondo, C. Gomez, S. J. Horvath, J. Clayton, D.G. Ando, E.E. Sercarz, and L. Hood. 1988. Restricted use of T cell receptor V genes in murine autoimmune encephalomyelitis raises possibilities for antibody therapy. *Cell.* 54:577–592.

29. Lai, M.Z., Y.J. Yang, L.K. Chen, and M.L. Gefter. 1990. Restricted V-(D)-J junctional regions in the T cell response to lambda repressor: identification of residues critical for antigen recognition. *J. Immunol.* 144:4851–4856.

30. Danska, J.S., A.M. Livingstone, V. Paragas, T. Ishihara, and C.G. Fathman. 1990. The presumptive CDR3 regions of both T cell receptor alpha and beta chains determine the T cell specificity for myoglobin peptides. *J. Exp. Med.* 172:27–33.

31. Jorgensen, J.L., U. Esser, B.F. de St.Groth, P.A. Reay, and M.M. Davis. 1992. Mapping T cell receptor-peptide contacts by variant peptide immunization of single chain transgenics. *Nature (Lond.).* 355:224–230.

32. Pierce, J.L., K.A. Zborowski, E. Kraig, and A.J. Infante. 1994. Highly conserved TCR beta chain CDR3 sequences among immunodominant acetylcholine receptor-reactive T cells in murine myasthenia gravis. *Int. Immunol.* 6: 775–783.

33. Heeger, P.S., W.E. Smoyer, T. Saad, S. Albert, C.J. Kelly, and E.G., Neilson. 1994 Molecular analysis of the helper T cell response in murine interstitial nephritis. T cells recognizing an immunodominant epitope use multiple T cell receptor V beta genes with similarities across CDR3. *J. Clin. Invest.* 94: 2084–2092.

34. Katayama, C.D., F.J. Eidelman, A. Duncan, F. Hooshmand, and S.M. Hedrick. 1995. Predicted complementarity determining regions of the T cell receptor determine antigen specificity. *EMBO J.* 14:927–938.

35. Bowditch, R.D., P.H. Tani, C.E. Halloran, A.L. Frelinger III, R. McMillan, and M.H. Ginsberg. 1992. Localization of a PlA1 epitope to the amino terminal 66 residues of platelet glycoprotein IIIa. *Blood.* 79:559–562.

36. Blanchette, V.S., L. Chen, Z.S. de Friedberg, V.A. Hogan, E. Trudel, and F. Decary. 1990. Alloimmunization to the PlA1 platelet antigen: results of a prospective study. *Br. J. Haematol.* 74:209–215.

37. Walchshofer, S., D. Ghali, M. Fink, E.R. Panzer-Grumayer, and S. Panzer. 1994. A rare leucine 40/arginine 40 polymorphism on platelet glycoprotein IIIa is linked to the human platelet antigen 1b. *Vox Sang.* 67:231–234.