

min and 30 cycles of denaturation at 95°C for 45 sec, annealing at 60°C for 1 min and extension at 72°C for 80 sec. The PCR cycling parameters for GPI-II and GPI-III were, an initial denaturation at 95°C for 2 min and 30 cycles of denaturation at 95°C for 45 sec, annealing at 60°C for 1 min and extension at 72°C for 2 min. A final extension at 72°C for 5 min was done in all the above reactions. Figure 9 is a EtBr stained agarose gels showing the PCR amplicons of the four partial mouse GPI gene .

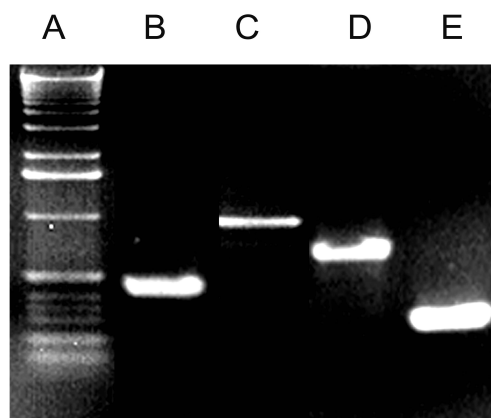


Figure 9. PCR amplification of truncated mouse GPI. (A) Marker. (B) GPI-I (amino acid residues 1-190). (C) GPI-II (amino acid residues 1-368). (D) GPI-III (amino acid residues 324-559). (E) GPI-IV (amino acid residues 485-559).

The PCR products were cloned into the TOPO vector, transformed into XL2-Blue cells and selected by blue/white selection. Positive clones were screened by double digestion of plasmids with EcoRI and XhoI to release the cloned fragments. The DNA insert bands were excised, purified, cloned into the GST vector pGEX-4T-3 and transformed into BL21 bacteria for expression.

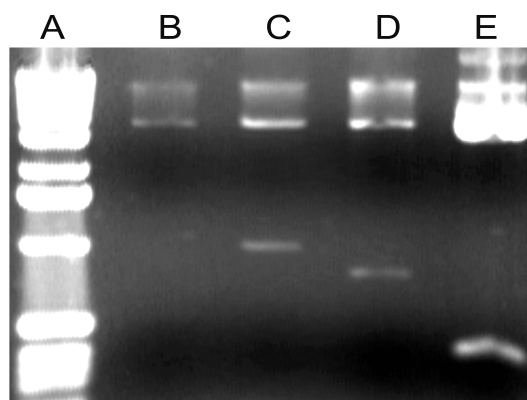


Figure 10. Screening of PGEX-4T3 plasmids for truncated mouse GPI inserts by restriction enzyme digestion analysis. (A) Marker. (B)GPI-I (1-190). (C) GPI-II (1-368). (D) GPI-II ((324-559). (E) GPI (485-559).

The clones were screened for recombinant protein expression by IPTG induction. Figure 11 shows a SDS-PAGE with the bacterial clones expressing the four GST-mouse GPI partial recombinant protein. All the clones expressed recombinant protein of the expected size. The expressed truncated versions of mouse GPI from these clones were used for epitope mapping by western blotting

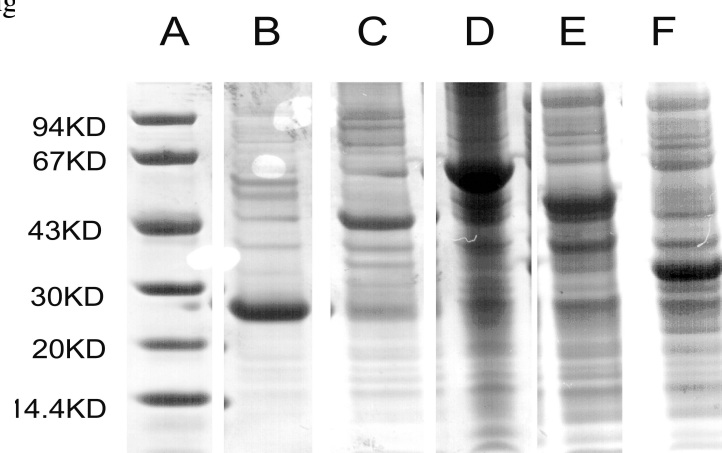


Figure 11. SDS-PAGE gel showing expression of truncated recombinant Mouse GPI in BL21 cells (A) Marker. BL21 Bacterial lysates expressing (B) GST alone. (C) GST-GPI-I(1-190). (D) GST-GPI-II (1-368). (E) GST-GPI-III (324-559). (F) GST-GPI-IV (485-559).

4.1.3. Cloning, expression and purification of recombinant human glucose-6-phosphate isomerase.

Human GPI was cloned by RT-PCR amplification, from IM9 human mature B-cell line total RNA, using the PCR primers, Forward-KN149 5'GGA GAA TTC TAT GGC CGC TCT CAC CCG GGA 3' and Reverse-KN150 5'TTC CTC GAG TAA GGG CTC GTG GTC CAA GC 3'. The PCR cycling parameters were an initial denaturation at 94°C for 2 min, followed 72°C for 4 min. A final extension at 72°C completed the amplification. Figure 12 shows the *Pfu* DNA polymerase amplified human GPI PCR amplicon.

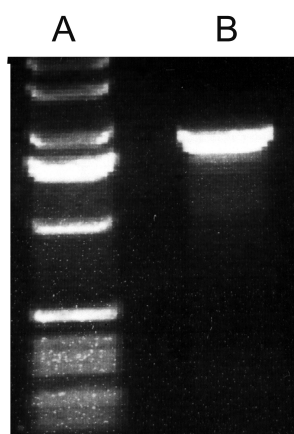


Figure 12. PCR amplification of human GPI gene. (A) Marker. (B) PCR amplified hGPI gene.

The PCR products were cloned into TOPO vector. The EcoRI and XhoI released DNA inserts excised from positive recombinant TOPO vectors were sub-cloned into linearized pGEX-4T-3 vectors. The recombinant pGEX-4T3 vectors screened of inserts for right size (figure 13), confirmed by DNA sequencing and transformed into BL21 *E. coli* for protein expression.

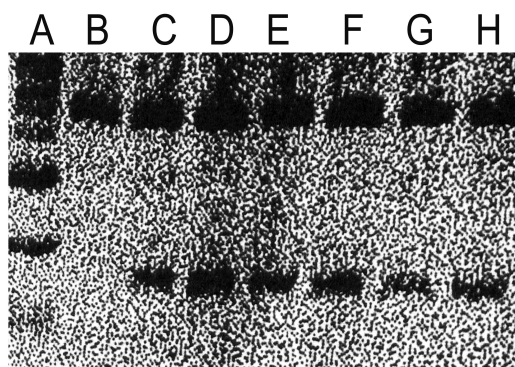


Figure 13. Screening of PGEX-4T3 plasmids for human GPI DNA inserts by restriction digestion analysis. (A) Marker. (B) Plasmid without any insert. (C-H) Plasmids with inserts.

The human GPI protein were induced and purified in a similar manner described for mouse GPI. The expression quality was similar to mouse GPI. Figure 14 shows the different stages of protein expression and purification of the recombinant human GPI. The glutathione affinity purified and thrombin cleaved human GPI was further purified by gel permeation chromatography before use. The recombinant protein was used to establish human GPI western blot and ELISA screening assays.

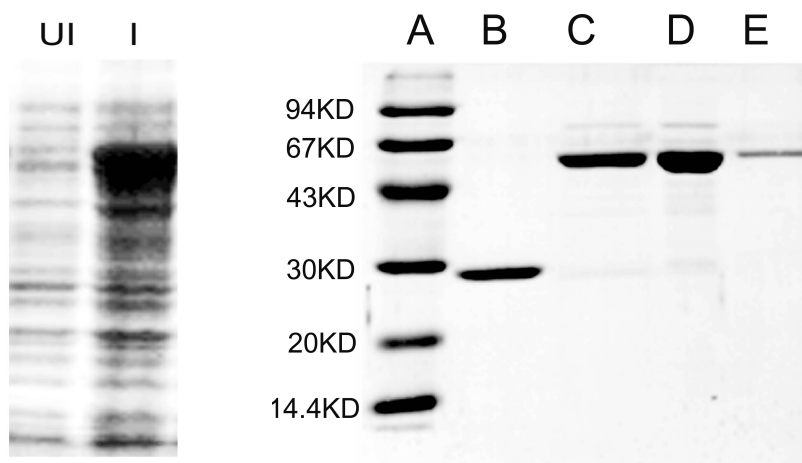


Figure 14. SDS-PAGE showing expression and purification of recombinant human GPI from BL21 cells. (UI) Uninduced bacterial lysate. (I) IPTG Induced bacterial lysate. (A) Marker. (B) Cleaved GST alone. (C, D) Glutathione affinity purified and thrombin cleaved hGPI protein. (E) Gel chromatography purified hGPI.

4.1.4. Glucose-6-phosphate isomerase reactive hybridomas cloned from un-manipulated K/BxN RA mice produced only IgM antibodies and were cross-reactive.

In order to generate GPI specific monoclonal hybridoma, a spleen from 1-year-old non-boostered arthritic K/BxN mice was dissected out. Single cell suspension from the spleen without any prior culture or stimulation was fused with the Sp2/0 myeloma cell line. The hybridoma fusion clones were screened for antibody production by recombinant mice GPI ELISA and immunoblotting (figure 15). Unusually high numbers of GPI reactive clones were found in the initial screens. However most of clones lost the GPI reactivity on sub-culture rapidly. The clones that were positive on GPI ELISA (using a polyclonal secondary antibody against mouse IgG heavy and light chain) were selected for further analysis. However on further screening for antibody isotypes it was found that all the clones only secreted antibodies of IgM type and the GPI ELISA screens had detected it because of the light chain reactivity. Stable positive clones with high reactivity were sub-cultured two or three times more. Some stable clones were chosen for sub-cloning by limiting dilution. All these IgM secreting clones also cross-reacted with GST, BSA and milk protein in ELISA (summarized in table 6) for this reason it was decided not to make use of these antibodies for further analysis.

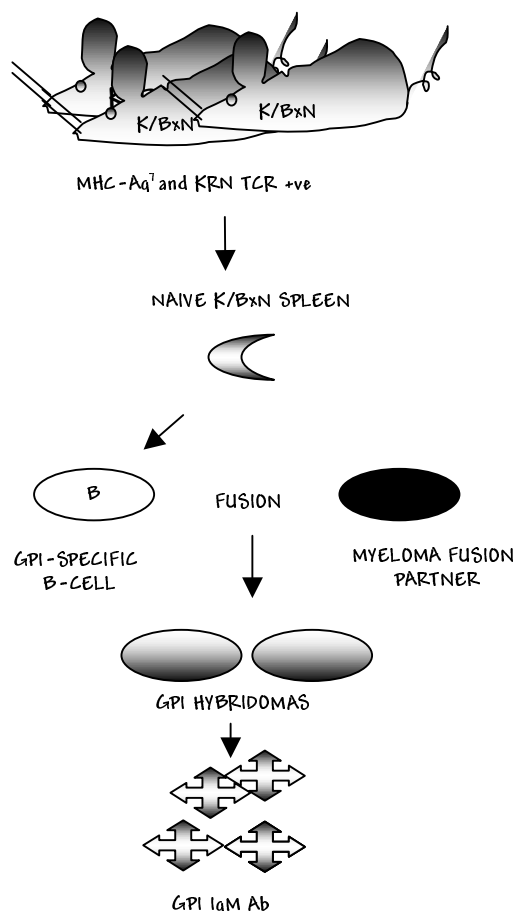


Figure 15. Illustration showing scheme for production of GPI monoclonal antibodies from naïve K/BxN mice.