This is to certify that the

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Phage Expressed Antibodies
to
Canine Erythrocyte Antigens

presented by

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John A. Gerlach

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Phage Expressed Antibodies to Canine Erythrocyte Antigens

By

Jonelle Karena Golding

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ABSTRACT

Phage Expressed Antibodies to Canine Erythrocyte Antigens

By

Jonelle Karena Golding

The construction of a human and canine phage expressed antibody library to erythrocyte antigens was attempted by the use of polymerase chain reaction (PCR) to amplify families of variable heavy and variable light chains. These amplified chains were consequently used in random pairing into single chain variable fragments (ScFv's). ScFv phage expression is the result of the insertion of the construct into a pCANTAB 5 E bacteriophage vector followed by transformation of competent *Escherichia coli* TG1 which is utilized for complete assembly and expression of the ScFv on the bacteriophage surface with the aid of M13K07 helper phage. Variable heavy and light chains were successfully amplified. Successful assembly and expression of human V_H5-$V_\lambda5$ ScFv was accomplished. The selection of this phage expressed antibody on human red blood cells yielded 5 phage expressed ScFv's whose subsequent specificities were indeterminable.

To my parents,

Keith and Fae Golding

for their unconditional love and support.

To Tracy A. Hammer,

a wonderful friend who is dearly missed

"Not a day goes by that I don't think of you and wish I

could see you smile"

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TABLE OF CONTANTS

List of Tables vi

List of Figures vii

Introduction 1

Materials and methods 21

Whole Blood Collection 21

B Lymphocyte Isolation 21

RNA Isolation 24

First Strand cDNA Synthesis 25

cDNA Synthesis Efficiency Test 26

Genomic DNA Isolation 28

PCR Amplification of V_H, V_κ and V_λ cDNA and Assembly of ScFv genes 29

Cloning of ScFv Gene Repertoires 36

Selection of Antigen Positive Recombinant Phage Antibodies 40

Determination of Specificity.....41

Results 48

Discussion 72

Recommendations 79

References 80

List of Tables

Table 1. Primary PCR Primers 32

Table 2. ScFv Linker Primers 33

Table 3. Restriction Site Primers 35

Table 4. Summary of Amplified cDNA Chains 49

Table 5. Results of Inhibition of Agglutination Test ..55

Table 6. ELISA os Expressed V_H5-$V_\lambda5$ 1, 2, and 3 56

Table 7. Summary of Secondary Panning Results 57

Table 8. Summary of Band Sizes (kD)- Western Blot Analysis 64

Table 9. Summary of Band Sizes (kD)- Coomassie Blue Staining 70

Table 10. Secondary Panning Results of V_H5-$V_\lambda5$ 3 71

List of Figures

Figure 1. The Antibody Molecule 3

Figure 2. Locations of CDR-1, CDR-2 and CDR-3 4

Figure 3. Light Chain Recombination 6

Figure 4. Heavy Chain Recombination 7

Figure 5. VH and VL Chain Amplification Primer Positions.....15

Figure 6. The Single Chain Variable Fragment (ScFv) ...16

Figure 7. The pCANTAB 5 E Map 17

Figure 8. Schematic Assembly of ScFv Gene Repertoire ..31

Figure 9. PCR Amplification of 1st Strand cDNA 48

Figure 10. V_H5-$V_\lambda5$ ScFv Repertoire with Restriction Sites 51

Figure 11. PCANTAB 5 E and V_H5-$V_\lambda5$ Repertoire Ligation .52

Figure 12. Growth of Colonies on SOBAG Plates 54

Figure 13. Western Blot Analysis V_H5-$V_\lambda5$ 1 59

Figure 14. Western Blot Analysis V_H5-$V_\lambda5$ 2 60

Figure 15. Western Blot Analysis V_H5-$V_\lambda5$ 3 61

Figure 16. Western Blot Analysis V_H5-$V_\lambda5$ 4 62

Figure 17. Western Blot Analysis V_H5-$V_\lambda5$ 5 63

Figure 18. Coomassie Blue Staining of V_H5-$V_\lambda5$ 1 65

Figure 19. Coomassie Blue Staining of V_H5-$V_\lambda5$ 2 66

Figure 20. Coomassie Blue Staining of V_H5-$V_\lambda5$ 3 67

Figure 21. Coomassie Blue Staining of V_H5-$V_\lambda5$ 4 68

Figure 22. Coomassie Blue Staining of V_H5-$V_\lambda5$ 5 69

INTRODUCTION

In order to defend itself from organisms which cause disease, the body is equipped with an immune system. Two classifications of the immune system exist. Innate or non-specific immunity, whereby naturally occurring defense mechanisms provide an unsuitable environment for invading organisms. Acquired or specific immunity emerges as the body recognizes and targets for elimination, foreign material (antigens) which have been successful in surviving innate immunity (Janeway, 1993).

Acquired immunity requires the action of B and T lymphocytes in addition to antigen presenting cells (APCs). T lymphocytes recognize antigens displayed on the surface of APCs and elicit the production of cytokines and cytotoxic T lymphocytes which kill the infected cells (Grey, *et al.*, 1989). B lymphocytes encounter antigen and differentiate to produce antibody specific for that antigen. The antibody molecule serves many roles in providing immunity. Firstly, they are involved in opsonization, the coating of antigens to promote their phagocytosis. In this process antigen-antibody complexes are formed between antigen and the antigen binding sites of the antibody. The remaining unbound

portion of the antibody, also known as the constant fragment, binds to a phagocyte and brings it in contact with the antigen (Winter and Milstein, 1991). A second biologic function of an antibody is neutralization of the invading pathogen. In addition, antibody bound to foreign cells activates the complement system which damages cell membranes and results in cellular destruction (Nossal, 1993).

The structure of an antibody molecule consists of two regions : 1) the constant (C) and 2) the variable (V) (Figure 1). The V region is the portion of the antibody that is specific to the antigen that has elicited its production (Edelman, *et al.*, 1969). The C domain is constant within its isotype and responsible for activation of complement and other biological functions (Winter & Milstein, 1991).

The V and C regions are each composed of heavy (H) and light (L) polypeptide chains (Figure 1). There are five classes of H chains, mu (μ), delta (δ), gamma (γ), alpha (α), and epsilon (ε); these classify the isotypes of antibodies by function as immunoglobulin M (IgM), IgD, IgG, IgA and IgE respectively. The L chains are of two classes, kappa (κ) and lambda (λ); which unlike the heavy chains do not reflect any difference in function (Amit, *et al.*, 1986). The variable H chains carry the binding activity of the antibody and though variable L chains are not required for

ntigen binding activity, they provide additional structure to the antigen binding surface (Winter, *et al.*, 1994).

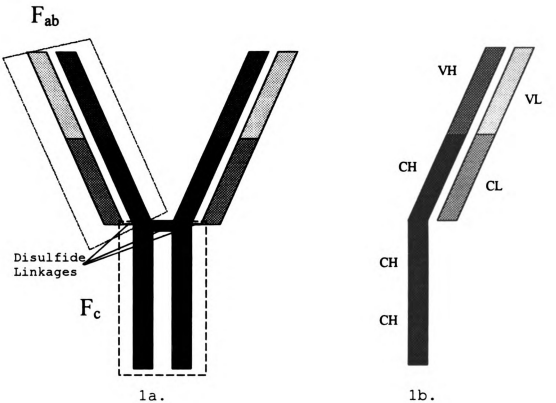

Figure 1. The Antibody Molecule.
1a. Antibody molecules are composed of Fab and Fc portions joined together by disulfide linkages.
1b. Each antibody molecule is composed of two heavy (H) chains and two light (L) chains. Portions of the H and L chains comprise the variable (V) region and the constant (C) region.

The antigen binding site is composed of two equal fragments each consisting of a variable region paired with a portion of the constant region with the two connected by disulfide linkages (Figure 1). The existence of these

fragments was demonstrated by papain cleavage to yield a crystallizable fragment (Fc) and a single antibody binding molecule known as the Fab (Cummings and Hooper, 1996).

The Fab portion of the antibody molecule binds specifically to the antigen which induced its production. Three hypervariable regions located in each VH and VL chain bind directly to the antigen. These hypervariable regions are designated as complementarity determining regions (CDR); CDR-1, CDR-2 and CDR-3 and are dispersed between invariable (framework) regions of the Fab (Figure 2). The immune system is able to generate diversity in antibodies by producing differing combinations of VH and VL regions (Barbas, 1995).

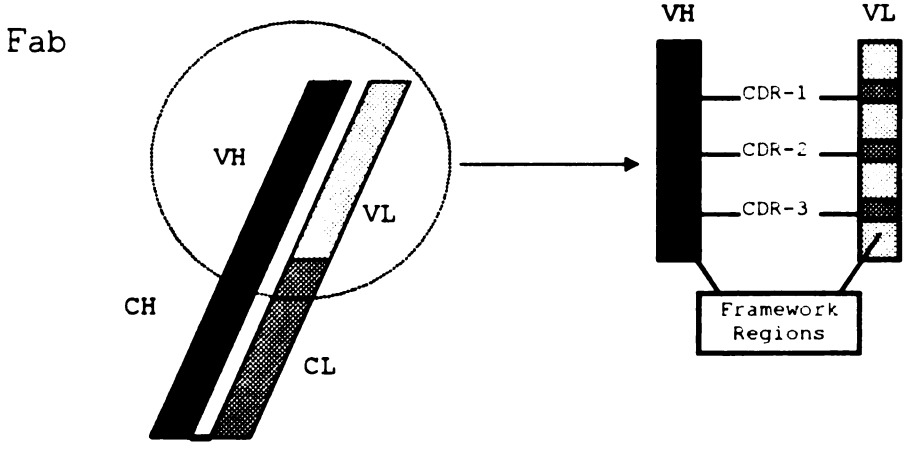

Figure 2. Locations of CDR-1, CDR-2 and CDR-3. Complementarity determining regions (CDR's) are hypervariable regions in the VH and VL chains and the portions of the variable regions which bind directly to antigen. They are located between framework regions which are invariable.

VH and VL recombinations produce a vast number of possibilities of antigen binding sites. In addition, multiple V region genes in the germ line and somatic mutation of germ lines provide an extensive diversity to antibody specificities (Tonegawa, 1983).

VL and VH chains result from recombinations of various gene segments which encode the CDR's. In the VL chain, CDR-1 and CDR-2 are encoded by the variable (V) segment and CDR-3 by the joining (J) segment, which is the site of joining between the V and C regions. In the human κ chain, it is estimated that there are 250 V and 5 J segments and for the λ, 2 V and 4 J segments. Recombination results from the joining of one V with one J segment (Figure 3). The process is permitted due to the existence of complementary, conserved nucleotide sequences which lie at the 3' end of the V and 5' end of the J segments. These sequences are brought together by a recombinase enzyme which contains 2 DNA binding proteins, each recognizing the complementary sequences. As the segments are joined the remaining segments are excised. Imprecise ends are produced from the joining of the segments and junctional diversity results from processing that provides for the addition of extra nucleic residues (P-nucleotides). This processing accommodates the joining of segments by filling the gaps that result from assymetric cleavage of the segments. The result of this

processing is the production of a VL chain from the recombination of the two gene segments (Tonegawa, 1983).

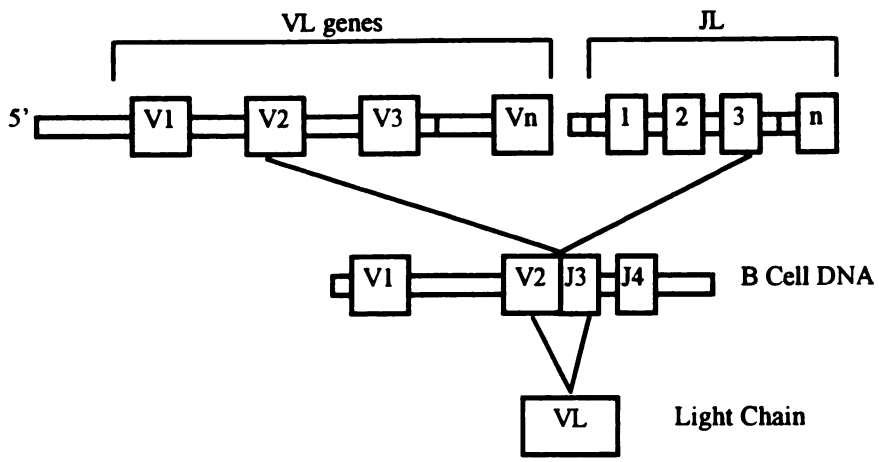

Figure 3. Light Chain Recombination.
The variable light chain is the result of the recombination of one V segment with a J segment.

The VH chain is composed of three gene segments. The CDR-1 and CDR-2 are encoded by the V segment and CDR-3 by the J and diversity (D) segment, which lies between the V and J segments. The estimation is that of are 200-1,000 V, 15 D and 4 J segments. The recombination of this chain occurs in two stages. The first joins the D and J segments, followed by the joining of the V to the DJ segment (Figure 4). The conserved nucleotide sequences for the V and J segments lie in the same positions they occur in the VL chain, and for the D segment are found at both ends. Junctional diversity in the VH chain is the result of P-

nucleotide addition, as well as a second process which utilizes the enzyme terminal deoxynucleotidyl transferase. This enzyme adds extra residues (N-nucleotides) not encoded by the template at the V to D and D to J joints. Both methods work together to produce a recombined VH chain (Tonegawa, 1983).

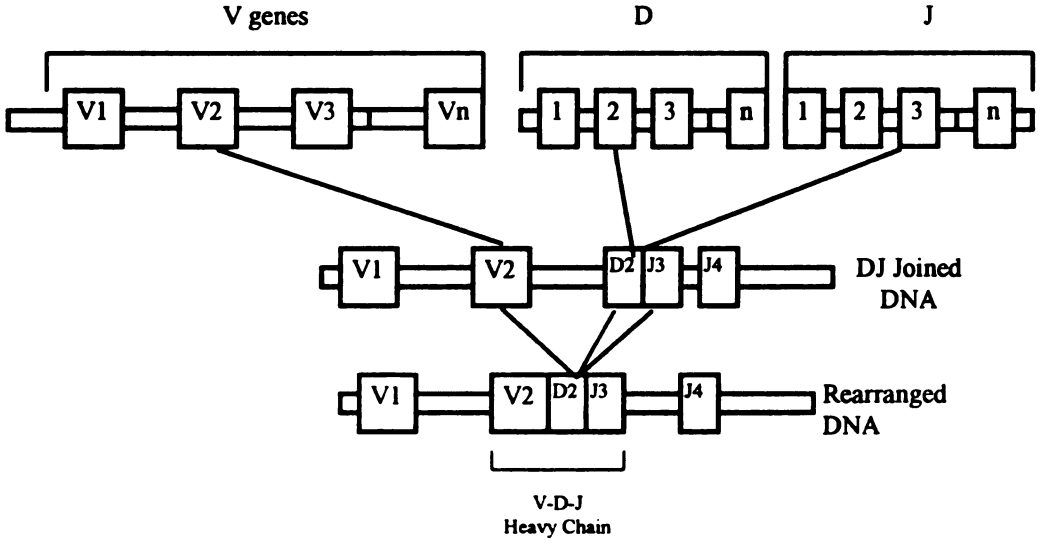

Figure 4. Heavy Chain Recombination
The variable heavy chain is the result of recombination of single V, D and J segments occuring in two stages. First, the D and J segments are joined to produce a DJ segment. This is followed by the joining of the V segment to the combined DJ segment.

The diverse number of antibodies that can be produced by the immune system because of the VDJ recombination, enables it to respond to a vast number of antigens presented to it. The effectiveness of this immune response is further enhanced by clonal selection, class switching and affinity

maturation. These processes allow the immune system to improve on the advantage of antibody production and provide faster, more efficient responses to invading pathogens.

Clonal selection provides the immune system with the capability to produce a proliferative antibody response that maintains its specificity to the initiating antigen. B lymphocytes circulate in the body with differing surface antibodies. When a B cell encounters an antigen to which its binding of the antigen is specific, the lymphocyte becomes activated and begins to divide. Each daughter cell that is produced, maintains the specificity of reaction that the initial cell possessed (Nossal, 1993).

Antibody producing B cells are also capable of switching the isotype of the antibody without alteration of its specificity. Each isotype of antibody provides different effector functions that contribute different advantages to the immune response. These functions include neutralizing toxins via IgG; protection of the nose, intestines and throat, provided by IgA; in addition to IgM providing first lines of defense. B cells switch classes of antibody in response to lymphokine production from T lymphocytes resulting in a more efficient response to the type of antigen the immune system is directing its attack against (Nossal, 1993).

Affinity maturation refers to the increased affinity of antibody for the inducing antigen that is seen over the course of an immune response. This phenomenon occurs late in the primary response to antigen and is seen more notably in secondary and following responses. As activated B cells proliferate, somatic hypermutation occurs and antigen binding by these cells takes place. Over time, cells with mutations with lower immunoglobulin binding affinities lose their binding capabilities. Cells with high affinity binding mutations continue to bind over time and the cell death is inhibited. This selective process increases the binding affinity of antibodies made over time (Jacob *et al.*, 1991).

Antibodies are used as tools in medicine and research because of the specificity of their interactions with antigens (Ansell, *et al,* 1996). Each cell type possesses specific, unique structures on their membrane that provide a physiological function and may characteristically differentiate them from each other. These discrete structures can also serve as antigens. Erythrocytes possess surface antigens that can be a barrier to transfusion. The identification of antigens on the erythrocyte surface provides necessary information that allows transfusion of compatible blood products to take place in order to avoid the production of immune clearance via opsonization (Ugozzoli and Wallace, 1992).

Science and technology have found alternative sources to produce antibodies for therapeutic and research purposes (Wigzell, 1993). The abilities of these antibodies to identify cell types render them useful and important diagnostic tools in clinical settings and as a result their production is a necessity to meet the demands of clinical laboratories (Matis and Rollins, 1995).

Stimulation of the immune system by presentation with antigen to produce an antibody specific to it is a commonly used technique in antibody production. This method however, results in polyclonal antibody production. Several clones responding to the same antigen with each having a slightly different recognition product. Therefore, though each antibody recognizes the antigen they are not the same. This method is not a feasible method to produce antibodies in humans. The practice of immunizing human subjects to produce desired antibodies that do not occur naturally presents an ethical dilemma. As a result other means of antibody production have been sought (Mayforth, 1993).

A second method of antibody production is the monoclonal antibody. This technique was first described by Kohler and Milstein in 1975. The process involves fusing of mouse splenic antibody producing cells with mouse myeloma cells, resulting in hybrid cells (hybridomas). Mouse myeloma cells are long-lived and produce stable hybrids. The

antibody producing cells need the long term capability of the myeloma cells. Selective media inhibits growth of unfused myeloma cells that lack the hypoxantihine:guanine phosphoribosyl transferase (HGPRT) enzyme genes, supplied to the hybridoma cells by the spleen cell. Unfused spleen cells die over time. The resulting, hybridoma cell clones are then selected for by antigen specificity. The use of this technology provides a continuous supply of antibody (Cummings and Hooper, 1996).

Monoclonal antibody production, though successful, has limitations. The antibodies are typically low-affinity because the technique does not allow for affinity maturation. These antibodies also produce an immunogenic response when used as therapeutic agents in humans as the constant region portion of the murine antibody induces an anti-murine antibody response (Winter & Milstein, 1991).

One response to the limitations of monoclonal antibody technology has been the development of chimeras, mouse-human antibodies. Using recombinant deoxyribonucleic acid (DNA) technology, mouse myeloma antibody genes encoding variable heavy and light regions from cells are joined with human constant heavy and light region genes. The rearranged genes are then transfected into the myeloma cell lines and selective media used to promote their growth. The result is the generation of cells transformed to produce antibody. If

seen in a human, there should be no response to the antibody as the constant region would not be recognized as non-human (Morrison, *et al.*, 1984).

Over time chimeras lose their ability to express the human immunoglobulin gene because of the selective loss of human chromosomes. Therefore a second modification of monoclonal antibody production, heteromyelomas, was developed. Heteromyelomas are mouse-human hybrid myelomas which use the longevity of mouse myeloma cells but allow the production of human monoclonal antibodies. Human B cells immortalized by transformation with Epstein-Barr virus (EBV) are fused with mouse-human hybridomas resulting in the production of stable human hybridomas that secrete human monoclonal antibodies (Teng, *et al.*, 1983).

Phage expressed antibodies have also emerged as an improved alternative method of antibody production. This technology enables the expression of the Fab fragments or the V domain of one Fab fragment, in the form of a single chain variable fragment (ScFv) on the surface of a filamentous bacteriophage (Peterson & Myones, 1996). The use of hybridoma technology and immunization to produce antibody is eliminated by this method. Phage expression of antibodies also has the capacity to combine original pairings of VH and VL chains in addition to any other combination. This provides the possibility of generating any combination of VH

and VL chains to produce any antigen binding site that may exist (Griffiths, *et al.*, 1993).

Filamentous bacteriophage are viruses with the capability of infecting male, gram negative bacteria and using the mechanisms available in these hosts for reproduction without causing cell lysis. Infection occurs by means of specific adsorption to the F pilus expressed on the surface of the bacteria. The bacteriophage adsorbs to the host by means of amino (N)-terminus of its minor coat protein, gene 3 protein (g3p) as the carboxy (C)-terminus remains anchored to the remainder of the bacteriophage coat. After attachment of phage to bacteria, a series of contractions, resulting from small localized charges in the pilus stimulate its retraction with the attached phage into the cell. Consequently, the phage DNA is released when the major coat protein, gene 8 protein (g8p) is removed and deposited on the host inner membrane. The bacteriophage uses host replication enzymes to produce a replicative form of its DNA, replicative form I (RFI) which acts as the template for phage replication. Viral gene 2 protein (g2p), ensures unit length production of newly synthesized viral strands (VS) and complementary strands (CS) of DNA. Host enzymes transcribe and translate the viral coat and replicative proteins. As this continues, the amount of gene 5 protein (g5p), increases and complexes with VS to hold them in

single strand(ss) conformation. Eventually, g2p synthesis ceases and a switch from replicative form (RF) DNA synthesis to ss viral DNA takes place. The g5p-VS complexes become associated with the g8p earlier deposited and expressed, in addition to other minor coat proteins, including the g3p. The result is phage progeny assembly as phage coat replaces g5p, encompassing the viral ss DNA. The host then cleaves the g3p and the phage is released (Rasched & Oberer, 1986).

The construction of phage expressed ScFv antibodies is the result of the polymerase chain reaction (PCR) amplification of the rearranged gene sequences encoding the VH and VL chains (Winter et al., 1994). The circulating B lymphocyte population is primarily CD19+ and have been shown to express original rearrangements of the VH chain (Demaison et al., 1995). The Vκ repertoire of this population have also been shown to undergo little or no somatic mutation (Klein et al., 1993). Therefore, peripheral B lymphocytes provide a reliable source of gene sequences of the V regions that are expressed as actual antigen binding sites.

PCR amplification utilizes primers that flank the V through J segments of VH and VL chains (Figure 5). These 5′ and 3′ primers classify the diverse repertoire of V genes into families. This classification is based on a conserved region within the families established on the basis of V gene sequences. The primer designs reflect the

classification of families and frequency of the most common nucleotide at each position (Marks, *et al.*, 1991).

Figure 5. VH and VL chain amplification primer positions. 5' and 3' primers classify V genes into families which share a conserved region and are used to amplify VH and VL chains for phage expression of ScFv.

The production of families of VH and VL chains separately provide the opportunity for original and random pairings in constructing the ScFv's. The result is the capability to produce an ScFv library with original antibody binding sites in addition to all the possible combinations of VH and VL. The diversity of antibody repertoires encoded in the V segment leads to the potential to produce antibody not expressed by the B cells in-vivo (Winter *et al.*, 1994).

The ScFv's, before being inserted into the phage genome are joined by PCR amplification to a linker in order to compensate for the omission of the C regions (Figure 6). The composition of the linker is glycine-glycine-glycine-glycine-serine-glycine-glycine-glycine-glycine-serine-glycine-glycine-glycine-glycine-serine. This $(Gly_4Ser)_3$

linker is a simple amino acid construct selected for its lack of secondary structure formation. This is an important feature to prevent the obstruction of the natural folding of the VH and VL chains necessary for the formation of the antigen binding sites (Huston et al., 1988).

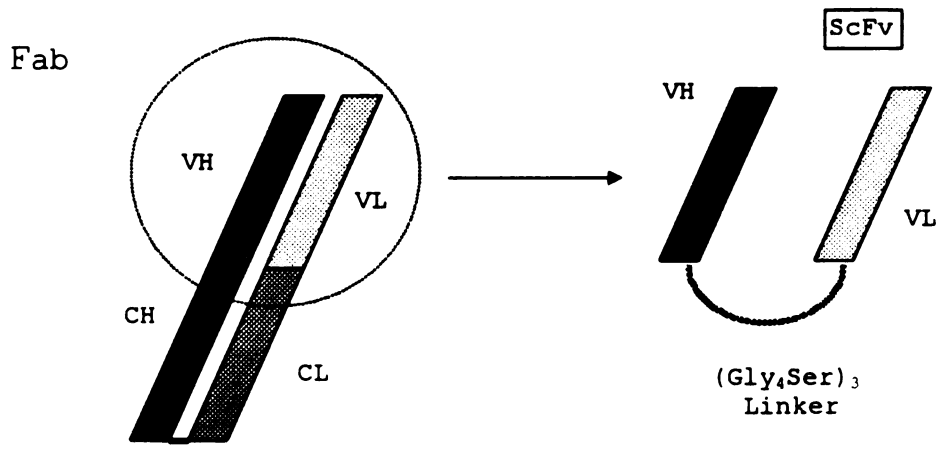

Figure 6. The single chain variable fragment (Scfv). The ScFv results from the combination of VH and VL genes of the Fab joined by a $(Gly_4Ser)_3$ linker.

The incorporation of the ScFv gene onto the surface of the bacteriophage for display requires that phage replication as previously described be interrupted for its insertion. This process utilizes the phagemid, pCANTAB 5 E, vector (Figure 7). This M13 filamentous bacteriophage possesses genes encoding ampicillin resistance, the stop codon amber, and *lac* promoter, all of which play an important role in successful insertion of the ScFv gene. It also possesses

genes encoding a peptide designated "E-tag", which is inserted prior to the stop codon and is used for the detection of phage expressed ScFv's. The ScFv is inserted into the g3p between the N and C termini which allows the immunologic display of the protein without alteration of the g3p infective ability (Smith, 1985). The bacteria, *E. coli* TG1, is infected with the pCANTAB 5 E vector containing the ScFv gene insert.

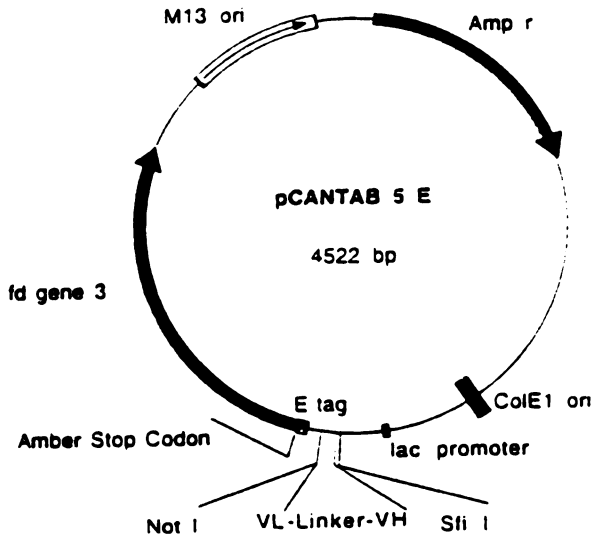

Figure 7. The pCANTAB 5 E map.
The locations of insertion point of the ScFV, origins of replication (M13 ori and ColE1 ori), E-tag, ampicillin resistance gene (Amp r), *lac* promoter and g3p as they are displayed in the phagemid genome are shown.
Translation with suppression of the amber codon due to the *supE* gene of *E. coli* TG1, permits the incorporation of the ScFv gene into the N-terminus g3p gene. Fusion of the ScFv gene to the N-terminus of the g3p allows the phage to retain

its infective ability, however the g3p accumulation is toxic to bacteria. In order to avoid toxicity, utilization of a helper phage, M13K07, into the system allows the phage to use the bacteria to incorporate the ScFv gene into the genome before g3p expression. pCANTAB 5 E carries an M13 origin of replication but lacks other coat proteins necessary for complete phage assembly. Infection of TG1 in the presence of glucose but absence of helper phage represses the *lac* operon to allow the phage to replicate with the protein insert but suppresses transcription and expression. Subsequent removal of glucose with simultaneous M13KO7 addition, induces the *lac* promoter to allow expression of the ScFv-g3p fusion protein on the surface of the phage. Soluble ScFv may be produced in the bacterial strain HB2151, which does not possess the ability to suppress the amber codon and results in termination at the E-tag to express only the ScFv-E-tag protein (Winter, *et al.*, 1994).

The successful construction of a variety of human phage displayed antibodies have been published. These include antibodies to influenza virus hemagglutinin (Caton and Koprowski, 1990), carcinoembryonic antigen (CEA) (Begent *et al.*, 1996), human immunodeficiency virus I (HIV-I) glycoprotein 120 (Burton, *et al.*, 1992), Hepatitis B surface antigen (HBsAg) (Zebedee, *et al.*, 1992) and cytomegalo virus

(CMV) (Williamson, *et al.*, 1993). Of particular interest were the phage expressed antibodies to red blood cell antigens of the ABO and Rh systems reported by Marks et al., 1993. Specifically these antibodies were made to B (ABO system) and D and E (Rh system).

Between mammals, it has been found that the response of the immune systems are similar with lymphocytes playing a major role. Canines and humans share these similarities. The immune responses of both species are specific and use B cells to produce antibody. The immunoglobulin classes produced by both are IgA, IgM and IgG however dogs have the tendency to produce primarily lambda L chain (Kehoe, 1982).

Similarities in the VH and VL chains of dogs and humans also exist. Human VH chains have been divided into six subgroups, V_HI-V_HVI. Comparisons of these subgroups with canine VH chains have shown a significant degree of homology between V_HIII and the canine VH chain (Kehoe and Capra, 1972). In addition, the comparison of V_κ chains between the species demonstrated a relationship between the canine V_κ chains and human V_κ-II of the six subgroups (Kehoe, 1982).

The red cell system in the canine and human species are also similar. Transfusion reactions, the result of IgG and IgM antibodies responding to cellular surface antigens occuring when incompatible blood is given, is seen in humans and dogs. The dog is also frequently used as a comparative

model to the human in diseases of red blood cell incompatibility and bleeding disorders (Bull, 1976). Similarities have also been established between dog erythrocyte antigen (DEA)-7 and the human ABO antigen A (Bowdler, *et al.*, 1973).

In humans, the major blood groups considered for transfusion compatibility are ABO and Rh. The ABO Blood types first introduced by Landsteiner in 1900 are A, B, AB and O. In the case of the Rh system, discovered by Levine and Stetson in 1939, an individual is either Rh positive or negative in addition to their ABO type.

Blood types in humans and canines are determined by agglutination typing with antisera containing monoclonal or polyclonal antibodies. Current methods of determining DEAs utilize antisera produced by immunization of dogs with small quantities of incompatible red cells to elicit an immune response and produce antisera. Due to the similarities expressed between human and canine immune systems, methods shown to be successful in producing human anti-erythrocyte antibodies may be successful for canine anti-erythrocyte antibody production. In search of locating a replacement canine anti-erythrocyte antibody production source, the goal of this thesis was to produce phage expressed ScFv's to the recognized canine erythrocyte antigens: DEA 1.1, 1.2, 3, 4, 5, 6, 7 and 8 (Vriesendorp, 1976).

MATERIALS AND METHODS

All reagents used in these methods, unless otherwise stated, were supplied by various biochemical and chemical manufacturers and purchased from Michigan State University Stores (East Lansing, Michigan).

Whole Blood Collection

Canine blood samples used in this section were pooled blood obtained from two dogs. One dog was DEA-4, the second DEA-4,7. Both animals were previously immunized and actively producing antibodies of anti DEA-1.1,2 specificity. Human blood samples were obtained from an ABO Type A1, individual producing anti-B and served as a positive control.

B Lymphocyte Isolation

All steps in this procedure were done under sterile conditions.

Forty milliliters (ml) of whole blood was collected in acid citrate dextrose (ACD) tubes and centrifuged at 750 x gravity (g) for 20 minutes (min). The buffy coat containing lymphocytes was transferred to a 15 ml conical polypropylene tube and diluted to three times the volume in phosphate buffered saline, pH 7.4 (PBS) (0.14 molar (M) sodium chloride (NaCl), 3 millimolar (mM) potassium chloride, 8 mM

sodium phosphate monobasic (NaH_2PO_4) and 1 mM potassium phosphate monobasic (KH_2PO_4)). The diluted cells were layered over ficoll-hypaque (R.I. 1.3570) at a ratio of 10 ml diluted blood to 3 ml ficoll-hypaque and centrifuged at 750 g for 10 min. The lymphocytes at the interface were removed and transferred to a new tube and washed once by diluting to 15 ml with PBS and centrifugation at 750 g for 20 minutes. The supernatant was discarded, the pellet resuspended in 15 ml Tris ammonium chloride (0.14 M ammonium chloride (NH_4Cl) and 6 mM Tris hydrochloride (Tris-HCl), pH 7.2) and incubated at 37°C for 10 min to lyse red blood cells. The suspension was centrifuged at 200 g for 10 min, the supernatant discarded and the pellet washed as described above. The supernatant was discarded and the pellet resuspended in 3 ml of cold 2% volume/volume (v/v) fetal calf serum (FCS, Sigma Chemical Co., St. Louis, Missouri) in PBS (PBS/FCS).

DYNABEADS® M-450 Pan-B (CD19) (Dynal, Inc., Lake Success, New York) magnetic beads were used to isolate B lymphocytes from the lymphocytes previously isolated. There were approximately 7.4×10^6 B lymphocytes in the 40 ml of peripheral blood to be isolated. Optimal isolation was accomplished by magnetic bead capture with 25 microliters (ul) of CD19 beads per 2.5×10^6 cells/ml of B lymphocytes (i.e., 75 ul of CD19 magnetic beads were used to capture the

7.4×10^6 B lymphocytes isolated). CD19 beads were prepared by dilution in 3 ml PBS/FCS, placed against a magnet for 2 min and the fluid removed. Beads were washed twice, each wash consisting of suspension of beads in 2 ml PBS/FCS, placing against a magnet for 2 min and removal of fluid. Beads were then resuspended in original volume of 75 ul and added to the lymphocyte suspension. The cell-bead suspension was incubated at 4 degrees Celsius (°C) for 15 min with rotation at "speed" 3 on a Multi-Purpose Rotator®, Model 150V (Scientific Instruments, Inc., Springfield, Massachussettes). The magnetic beads with B lymphocytes attached were then placed against a magnet for 1 min, fluid removed and washed once as previously described. The bead/cells were resuspended in 100 ul PBS/FCS and 10 ul DETACHaBEAD® (Dynal, Inc., Lake Success, New York) added to detach B lymphocytes from beads. The mixture was incubated for 60 min at room temperature with gentle agitation at 10 minute intervals. 1 ml PBS/FCS was added to suspension, the tube was placed against a magnet for 2 min and fluid with B lymphocytes transferred to 1.5 ml conical polypropylene microcentrifuge tube. B lymphocytes were pelleted by centrifugation at 750 g for 10 min. Cells were washed twice in 1 ml PBS with centrifugation at 2,000 g for 2 min.

Ribonucleic Acid (RNA) Isolation

All steps of RNA isolation were performed under sterile conditions and all surface areas and pipets used were cleaned with RNAse Free® (Continental Laboratory Products, Inc., San Diego, California) before use in the procedure.

1 ml of TRI REAGENT® (Molecular Research Center, Inc., Cincinatti, Ohio) was added to a pellet of 7.4×10^6 B lymphocytes washed once with diethyl pyrocarbonate (DEPC) treated PBS (0.2% v/v) (DEPC-PBS) and incubated for 5 min at room temperature (RT) to solubilize the cells. 0.2 ml of chloroform/isoamyl alcohol (24:1 v/v) was added to the homogenate and vigorously shaken for 15 seconds (sec). The sample was incubated for 15 min at RT and centrifuged at 10,000 g for 15 min at 4°C. The upper aqueous phase containing RNA was transferred to a clean 1.5 ml centrifuge tube and the RNA precipitated by mixing with 0.5 ml of isopropanol. Samples were incubated at room temperature for 10 min and centrifuged at 10,000 g for 10 min at 4°C to pellet RNA. The supernatant was discarded and the pellet washed once in DEPC treated water (0.2% v/v) (DEPC-HOH) with 75% ethanol. The supernatant was discarded and the pellet air dried for 15 minutes and resuspended in 20 ul DEPC treated 3M sodium acetate with 70% ethanol, pH 5.2. The RNA

was frozen at -70°C until the next procedure was to be performed.

First Strand Complementary Deoxyribonucleic acid (cDNA) Synthesis

First strand cDNA synthesis was performed under sterile conditions and all reagents were supplied with the 1st-strand™ cDNA Synthesis Kit (Clonetech Laboratories, Inc., Palo Alto, California).

7.5 ul of RNA and 5 ul DEPC-HOH were mixed on ice and 1 ul of random hexamer primer (20 micromolar (uM)) added. The sample was heated for 2 min at 70°C in a DNA Thermal Cycler® (Perkin Elmer Cetus, Norwalk, Connecticut) and immediately placed on ice. A mixture of 1) 4 ul 5X Reaction Buffer (250 mM Tris-HCL, pH 8.3), 375 mM potassium chloride (KCl) and 15 mM magnesium chloride ($MgCl_2$), 2) 1 ul deoxynucleotide 5'-triphosphate (dNTP) mix (10 mM each deoxyadenosine 5'-triphosphate (dATP), deoxycytidine 5'-triphosphate (dCTP), deoxyguanosine 5'-triphosphate (dGTP) and deoxythymidine 5'-triphosphate (dTTP), 3) 0.5 ul recombinant ribonuclease (RNase) inhibitor (40 U/ul) and 4) 1 ul Moloney-Murine Leukemia Virus recombinant (MMLV) reverse transcriptase (200 U/ul) was added to the 13.5 ul reaction mixture and incubated at 42° C for 60 min followed by 94°C for 5 min in DNA Thermal Cycler® (Perkin Elmer Cetus, Norwalk,

Connecticut). The tubes were then centrifuged at 10,000 g for 30 sec and efficiency of synthesis tested.

cDNA Synthesis Efficiency Test

The efficiency of the first strand cDNA synthesis was tested by polymerase chain reaction (PCR) amplification of cDNA with primers to amplify a 540 base pair (bp) band of human β-Actin. β-Actin is used as a marker due to the presence of its messenger RNA (mRNA) in high quantities in most cells. The sequences for the primers were obtained from Human β-Actin Control Amplimer Set (Clonetech Laboratories, Inc., Palo Alto, California) and synthesized at Michigan State University Macromolecular Structure Facility (East Lansing, Michigan). The sequences are as follows: 5' primer: 5'ATC TGG CAC CAC ACC TTC TAC AAT GAG CTG CG 3'; 3' primer: 5' CGT CAT ACT CCT GCT TGC TGA TCC ACA TCT GC 3'. Primers were prepared by vaccum drying on high for 45 min in Savant Speed Vac SC100® (Savant Instruments, Inc., Farmingdale, New York) and reconstituting in double-distilled water (ddHOH) to final concentration of 20 mM.

The PCR reaction mixture consisted of 1) 2.5 ul cDNA (diluted 1:5 with DEPC-HOH), 2) 0.5 ul each dATP, dCTP, dGTP, dTTP (10 mM), 3) 1.25 ul each 5' and 3' primer (20 uM), 4) 0.1 ul *Thermus aquaticus* (AmpliTaq®) DNA polymerase (5 U/ul) (Perkin Elmer Cetus, Norwalk, Connecticut), 5) 2.5

ul GeneAmp® 10X PCR buffer (15 mM $MgCl_2$, 500 mM KCl, 100 mM Tris-HCl (pH 8.3) and 0.01% (w/v) gelatin) (Perkin Elmer Cetus, Norwalk, Connecticut) and 6) 15.4 ul DEPC-HOH. Components 2-6 were made in a master mix and 22.5 ul added to cDNA in each reaction. The parameters of the reaction were 1) 94°C for 45 sec denaturing, 2) 60°C for 45 sec annealing and 3) 72°C for 1 min extension each cycle for 30 cycles in GeneAmp PCR System 9600® (Perkin Elmer Cetus, Norwalk, Connecticut). 20 ul of amplified product was mixed with 5 ul 6X loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol and 40% weight/volume (w/v) sucrose in water), loaded on a 2% (w/v) SeaKem GTG® (FMC Bioproducts, Rockland, Maine) agarose gel in 1X Tris-borate ethylenediaminetetraacetic acid disodium salt (EDTA) buffer (TBE) (0.89 M Tris base, 0.89 M boric acid and 0.002 M EDTA) and electrophoresed in 1X TBE buffer at 160 volts (V) for 45 min. The gel was stained by incubation in 0.5 ug/ml ethidium bromide in ddHOH for 15 min and cDNA bands visualized by trans-illumination with ultraviolet (UV) light using Chromato-vue Transilluminator (San Gabriel, California). The gel was photographed using type 667 film (Polaroid, Cambridge, Massachusetts). Upon observance of a band at 540 bp, the cDNA was used for PCR amplification of VH and VL chains.

Genomic DNA Isolation

PCR amplification of VH and VL chains was also performed using genomic DNA. Isolation of genomic DNA from B lymphocytes was performed under sterile conditions. B lymphocytes were transferred to a 15 ml tube and washed once by resuspending the cells in 1 ml Dulbecco's PBS (DPBS) without magnesium and calcium and centrifugation at 225 g for 20 min. The supernatant was discarded and the cells resuspended in a mixture of 2.25 ml of lysing solution (10 mM Tris (pH 7.6), 25 mM EDTA (pH 8.0) and 75 mM NaCl), 3.38 ul Proteinase K (50 ug/ml) and 42.19 ul 20% (w/v) sodium dodecyl sulfate (SDS). The mixture was incubated at 37°C for 2 hours (hr) followed by the addition of 22.5 ul of Ribonuclease A (RNase) (10 mg/ml). The tube was incubated at 37°C for 1 hr and 750 ul of 6M NaCl added. The tube was vigorously shaken for 10 seconds and centrifuged at 850 g for 10 min. The supernatant was transferred to a clean tube and centrifuged for 10 min at 850 g. The supernatant was transferred to a 15 ml polystyrene tube, 5.4 ml absolute ethanol (-20°C) added and the mixture gently inverted until a white precipitant formed. The precipitant was transferred to a 1.5 ml polypropylene tube, centrifuged at 10,000 g for 2 min, the supernatant discarded and the pellet washed twice with 1.5 ml 90% ethanol. Each wash consisted of centrifugation at 10,000 g for 2 min followed by discarding

the supernatant. The pellet was then dried under vaccum and resuspended in 100 mM Tris-HCl (pH 7.6) to a concentration of 0.1 ug/ul. The amount of DNA was determined by spectrophotometry (Maniatis, *et al.*, 1989).

PCR Amplification of V_H, V_κ and V_λ cDNA and Assembly of ScFv genes

The sequences of the primers used in these reactions were obtained from Marks, *et al.*, 1991 and synthesized at Michigan State University Macromolecular Structure Facility. All PCR amplification reactions were performed in a GeneAmp PCR System 9600® and electrophoreses were performed using a Bio-Rad Model 200/2.0 Power Supply® (Bio-Rad Laboratories, Hercules, California).

The V_H, V_κ and V_λ cDNA were used to create two ScFv gene repertoires, V_H-V_λ and V_H-V_κ (Figure 8). The primary PCR reaction was used to separately amplify V_H, V_κ and V_λ cDNA utilizing the HuVHBACK, HuJHFOR, HuVLBACK and HuJLFOR primers in Table 1 (See Figure 8a). The PCR reaction mixtures consisted of 2.5 ul genomic DNA or first strand cDNA, 2.5 ul GeneAmp® 10X PCR Buffer, 1.25 ul each forward and reverse primer, 0.5 ul each dATP, dCTP, dGTP and dTTP, 0.1 ul AmpliTaq® DNA polymerase and 15.4 ul ddHOH. PCR amplification parameters were 94°C for 45 sec denaturing,

60°C for 45 sec annealing and 72°C for 1 min extension each cycle for 30 cycles. 5 ul of product was mixed with 1 ul 6X loading buffer and electophoresed on an 8% polyacrylamide gel (16.4 mM acrylamide, 0.2 mM bis (N,N'-methylene-bis-acrylamide) in 1X Tris-acetate EDTA (TAE) buffer (0.08 M Tris-HCl, 4 mM EDTA and 0.23% glacial acetic acid) at 160V for 30 min. Staining, visualization and photography were performed as previously described. The linker DNA was prepared by 52 separate amplifications of the $(Gly_4Ser)_3$ linker using each of the reverse JH primers in combination with each reverse V_κ and V_λ primers (Table 2). The PCR mixture used was 2.5 ul GeneAmp® 10X PCR Buffer, 1.25 ul each forward and reverse primer, 1.25 ul each dATP, dCTP, dGTP and dTTP, 0.25 ul AmpliTaq® DNA polymerase 15.4 ul ddHOH and 0.5 ul of linker DNA (20 uM). PCR amplification parameters were 94°C for 1 min denaturing, 60°C for 30 sec annealing and 72°C for 1 min extension each cycle for 30 cycles. 5 ul of product was loaded with 1 ul 6X loading buffer on to an 8% polyacrylamide gel and electrophoresed, stained and photographed as described in the primary PCR reactions. The 52 separate PCR products were then pooled using equal volumes and used as one template for ScFv assembly.

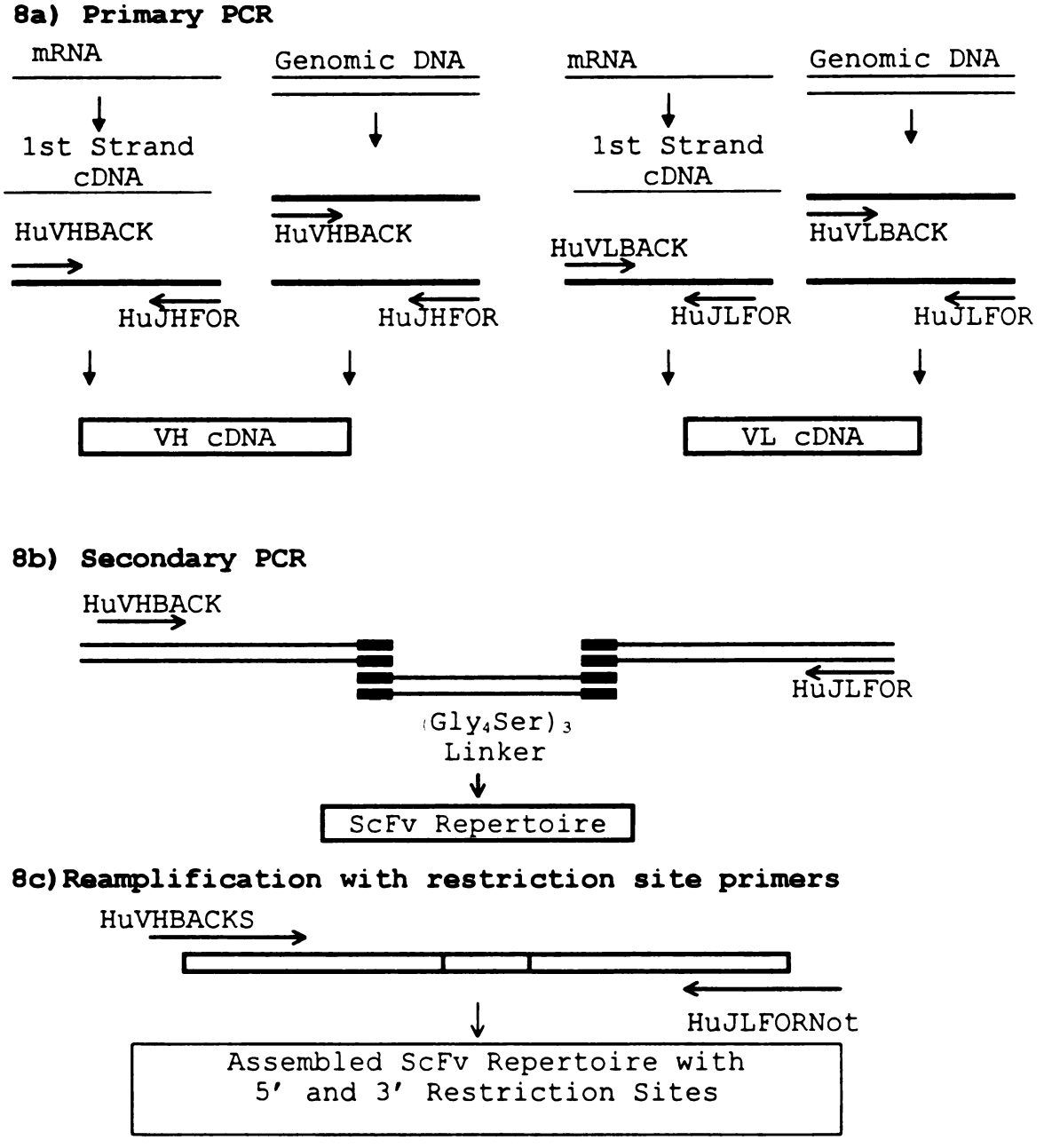

Figure 8. Schematic Assembly of ScFv Gene Repertoire
(a) 1st strand cDNA produced from PCR amplification of mRNA or genomic DNA is amplified with VBACK and JFOR primers to produce VH and VL cDNA. (b) VH and VL PCR products are combined in second amplification process with linker DNA to generate ScFv repertoires. (c) ScFv gene repertoires are reamplified with primers containing restriction sites to produce assemble ScFv repertoire with restriction sites.

Table 1. Primary PCR Primers.
Primers used for the generation of V_H, V_κ and V_λ cDNA.

Human V_H back primers.

HuVH1aBACK	5'-CAG GTG CAG CTG GTG CAG TCT GG-3'
HuVH2aBACK	5'-CAG GTC AAC TTA AGG GAG TCT GG-3'
HuVH3aBACK	5'-GAG GTG CAG CTG GTG GAG TCT GG-3'
HuVH4aBACK	5'-CAG GTG CAG CTG CAG GAG TCG GG-3'
HuVH5aBACK	5'-GAG GTG CAG CTG TTG CAG TCT GC-3'
HuVH6aBACK	5'-CAG GTA CAG CTG CAG CAG TCA GG-3'

Human J_H forward primers.

HuJH1-2FOR	5'-TGA GGA GAC GGT GAC CAG GGT GCC-3'
HuJH3FOR	5'-TGA AGA GAC GGT GAC CAT TGT CCC-3'
HuJH4-5FOR	5'-TGA GGA GAC GGT GAC CAG GGT TCC-3'
HuJH6FOR	5'-TGA GGA GAC GGT GAC CGT GGT CCC-3'

Human V_κ back primers.

Huκ1aBACK	5'-GAC ATC CAG ATG ACC CAG TCT CC-3'
Huκ2aBACK	5'-GAT GTT GTG ATG ACT CAG TCT CC-3'
Huκ3aBACK	5'-GAA ATT GTG TTG ACG CAG TCT CC-3'
Huκ4aBACK	5'-GAC ATC GTG ATG ACC CAG TCT CC-3'
Huκ5aBACK	5'-GAA ACG ACA CTC ACG CAG TCT CC-3'
Huκ6aBACK	5'-GAA ATT GTG CTG ACT CAG TCT CC-3'

Human J_κ forward primers.

HuJκ1FOR	5'-ACG TTT GAT TTC CAC CTT GGT CCC-3'
HuJκ2FOR	5'-ACG TTT GAT CTC CAG CTT GGT CCC-3'
HuJκ3FOR	5'-ACG TTT GAT ATC CAC TTT GGT CCC-3'
HuJκ4FOR	5'-ACG TTT GAT CTC CAC CTT GGT CCC-3'
HuJκ5FOR	5'-ACG TTT AAT CTC CAG TCG TGT CCC-3'

Human λ back primers.

Huλ1BACK	5'-CAG TCT GTG TTG ACG CAG CCG CC-3'
Huλ2BACK	5'-CAG TCT GCC CTG ACT CAG CCT GC-3'
Huλ3aBACK	5'-TCC TAT GTG CTG ACT CAG CCA CC-3'
Huλ3bBACK	5'-TCT TCT GAG CTG ACT CAG GAC CC-3'
Huλ4BACK	5'-CAC GTT ATA CTG ACT CAA CCG CC-3'
Huλ5BACK	5'-CAG GCT GTG CTC ACT CAG CCG TC-3'
Huλ6BACK	5'-AAT TTT ATG CTG ACT CAG CCC CA-3'

Human λ forward primers.

HuJλ1FOR	5'-ACC TAG GAC GGT GAC CTT GGT CCC-3'
HuJλ2-3FOR	5'-ACC TAG GAC GGT CAG CTT GGT CCC-3'
HuJλ4-5FOR	5'-ACC TAA AAC GGT GAG CTG GGT CCC-3'

Table 2. ScFv Linker Primers.
Primers used for generation of ScFv compatible ends and V_H, V_κ and V_λ cDNA.

Reverse J_H for ScFv linker	
RHuJH1-2	5'-GCA CCC TGG TCA CCG TCT CCT CAG GTG G-3'
RHuJH3	5'-GGA CAA TGG TCA CCG TCT CTT CAG GTG G-3'
RHuJH4-5	5'-GAA CCC TGG TCA CCG TCT CCT CAG GTG G-3'
RHuJH6	5'-GGA CCA CGG TCA CCG TCT CCT CAG GTG C-3'
Reverse V_κ for ScFv linker	
RHuVκ1aBACKFv	5'-GGA GAC TGG GTC ATC TGG ATG TCC GAT CCG CC-3'
RHuVκ2aBACKFv	5'-GGA GAC TGA GTC ATC ACA ACA TCC GAT CCG CC-3'
RHuVκ3aBACKFv	5'-GGA GAC TGC GTC AAC ACA ATT TCC GAT CCG CC-3'
RHuVκ4aBACKFv	5'-GGA GAC TGG GTC ATC ACG ATG TCC GAT CCG CC-3'
RHuVκ5aBACKFv	5'-GGA GAC TGC GTG AGT GTC GTT TCC GAT CCG CC-3'
RHuVκ6aBACKFv	5'-GGA GAC TGA GTC AGC ACA ATT TCC GAT CCG CC-3'
Reverse V_λ linker	
RHuVλBACK1Fv	5'-GGC GGC TGC GTC AAC ACA GAC TGC GAT CCG CCA CCG CCA GAG-3'
RHuVλBACK2Fv	5'-GCA GGC TGA GTC AGA GCA GAC TGC GAT CCG CCA CCG CCA GAG-3'
RHuVλBACK3aFv	5'-GGT GGC TGA GTC AGC ACA TAG GAC GAT CCG CCA CCG CCA GAG-3'
RHuVλBACK3bFv	5'-GGG TCC TGA GTC AGC TCA GAA GAC GAT CCG CCA CCG CCA GAG-3'
RHuVλBACK4Fv	5'-GGC GGT TGA GTC AGT ATA ACG TGC GAT CCG CCA CCG CCA GAG-3'
RHuVλBACK5Fv	5'-GAC GGC TGA GTC AGC ACA GAC TGC GAT CCG CCA CCG CCA GAG-3'
RHuVλBACK6Fv	5'-TGG GGC TGA GTC AGC ATA AAA TTC GAT CCG CCA CCG CCA GAG-3'

To assemble the ScFv genes the PCR amplification took place in two stages (Figures 8b and 8c). In the first portion of the reaction 5 ul of each VH and each VL cDNA were combined with 5 ul of linker DNA, 2.5 ul GeneAmp® 10X PCR Buffer, 1.25 ul each dNTP and 0.25 ul AmpliTaq® DNA polymerase. The mixture was amplified for 10 cycles (94°C for 2 min and 65°C for 2.5 min each cycle) to join the fragments together. To each mixture, 1.25 ul of the corresponding HuVHBACK and HuJLFOR primers each were added and the mixture cycled at 94°C for 1 min and 65°C for 3 min each cycle for an additional 30 cycles. 5 ul of product was mixed with 1 ul 6X loading buffer and electrophoresed on an 8% polyacrylamide gel, stained, visualized and photographed. The final step in the process was to add restriction sites on to the ScFv gene (See Figure 8c). 2.5 ul of assembled ScFv gene template with 0.5 ul each dNTP, 2.5 ul GeneAmp® 10X PCR Buffer, 15.4 ul HOH and 0.1 ul AmpliTaq® DNA polymerase was added to 1.25 ul each of the corresponding restriction site primers (Table 3). The mixtures were amplified at 94°C for 1 min denaturing, 55°C for 1 min annealing and 72°C for 2.5 min extension each cycle for 25 cycles. This was followed by electrophoresis, staining, visualization and photography of 5 ul of product with 1 ul of 6X loading buffer on 8% polyacrylamide gel.

Table 3. Restriction Site Primers
Primers used to generate the restriction sites on 5′ and 3′ ends of ScFv repertoire.

Human V_H Back Primers

HuVH1aBACKSfi	5′-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG GTG CAG CTG GTG CAG TCT GG-3′
HuVH2aBACKSfi	5′-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG GT AAC TTA AGG GAG TCT GG-3′
HuVH3aBACKSfi	5′-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC GAG GTG CAG CTG GTG GAG TCT GG-3′
HuVH4aBACKSfi	5′-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG GTG CAG CTG CAG GAG TCG GG-3′
HuVH5aBACKSfi	5′-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG GTG CAG CTG TTG CAG TCT GC-3′
HuVH6aBACKSfi	5′-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG GTA CAG CTG CAG CAG TCA GG-3′

Human J_κ Forward Primers

HuJκ1BACKNot	5′-GAG TCA TTC TCG ACT TGC GGC CGC ACG TTT GAT TTC CAC CTT GGT CCC-3′
HuJκ2BACKNot	5′-GAG TCA TTC TCG ACT TGC GGC CGC ACG TTT GAT CTC CAG CTT GGT CCC-3′
HuJκ3BACKNot	5′-GAG TCA TTC TCG ACT TGC GGC CGC ACG TTT GAT ATC CAC TTT GGT CCC-3′
HuJκ4BACKNot	5′-GAG TCA TTC TCG ACT TGC GGC CGC ACG TTT GAT CTC CAC CTT GGT CCC-3′
HuJκ5BACKNot	5′-GAG TCA TTC TCG ACT TGC GGC CGC ACG TTT AAT CTC CAG TCG TGT CCC-3′

HuJ_λ Forward Primers

HuJλ1FORNOT	5′-GAG TCA TTC TCG ACT TGC GGC CGC ACC TAG GAC GGT GAC CTT GGT CCC-3′
HuJλ2-3FORNOT	5′-GAG TCA TTC TCG ACT TGC GGC CGC ACC TAG GAC GGT CAG CTT GGT CCC-3′
HuJλ4-5FORNOT	5′-GAG TCA TTC TCG ACT TGC GGC CGC ACY TAA AAC GGT GAG CTG GGT CCC-3′

Cloning of ScFv Gene Repertoires

Digestion of the assembled ScFv product was preformed to generate compatible ends for insertion into the pCANTAB 5 E vector. 5 ul of ScFv product, 12 ul of sterile ddHOH, 2 ul of 10X One-Phor-All Plus (OPA) Buffer Plus (100 mM Tris-acetate, pH 7.5, 100 mM magnesium acetate and 500 mM potassium acetate) (Pharmacia Biotech, Piscataway, New Jersey) and 1 ul *Sfi* (10,000 U/ml) (Pharmacia Biotech, Piscataway, New Jersey) were mixed and 5 ul mineral oil layered over the mixture. The sample was incubated overnight at 50°C followed by inactivation at 85°C for 30 min. 0.8 ul *NotI* (12,000 u/ml) (Pharmacia Biotech, Piscataway, New Jersey) was added and incubated at 37°C overnight with 65°C inactivation for 20 min. 5 ul of 0.1% (v/v) Triton X-100 and 100 mM NaCl was added and 5 ul of the final product mixed with 1 ul 6X loading buffer and loaded on to an 8% polyacrylamide gel, electrophoresed, stained and photographed. Upon visualization of the appropriate size band to indicate successful digestion, the product was used for ligation.

Quantitation be gel electrophoresis of the assembled ScFv gene repertoire was performed before ligation into the pCANTAB 5 E vector to optimize the reaction using the appropriate quantities. A 1% SeaKem GTG® agarose gel with 0.5 ug/ml ethidium bromide was used. Control ScFv Marker

(Pharmacia Biotech, Piscataway, New Jersey) was loaded on the gel in 25 ng and 12.5 ng quantities. 2.5 ul and 5 ul of assembled ScFv product were loaded for comparison. The gel was electrophoresed in 1X TAE buffer as described previously and photographed. Upon visual comparison with the Control ScFv marker an estimate of the quantity of assembled ScFv was determined.

150 ng of assembled ScFv repertoire was ethanol precipitated by the addition of 125 ul of absolute ethanol and 5 ul 3 M sodium acetate, incubation at -20°C for 30 min and centrifugation 10,000 g for 20 min. The supernatant was discarded and the pellet resuspended in 300 ul 70% ethanol and centrifuged at 10,000g for 20 min. The pellet was vaccum dried with no heat for 2 min. 10 ul of ligation reaction mixture (1 ul T4 DNA ligase (5 U/ul) (Boehringer Mannheim, Indianapolis, Indiana), 5 ul 10X OPA Buffer, 5 ul pCANTAB 5 E (250 ng) (Pharmacia Biotech, Piscataway, New Jersey), 5 ul 10mM adenosine 5'-triphosphate (ATP), 39 ul sterile HOH) was added to the pellet and incubated for 4 hr at 16°C. The remaining 40 ul of ligation mixture was added and the sample incubated at 16°C overnight. Inactivation for 10 min at 70°C was performed and 5 ul of sample loaded with 1 ul 6X loading buffer on to 0.6% SeaKem GTG® agarose gel containing 0.5 ug/ml ethidium bromide and electrophoresed for 4 hr at 80V in 1X TAE buffer. The insertion of the assembled ScFv

repertoire into the vector was determined by photography and phage infection performed.

All steps of the phage infection procedure were performed under sterile conditions.

Competent *Escherichia coli* TG1 bacteria was used for transformation. Preparation of competent *E. coli* TG1 culture was performed by resuspending lyophilized culture in 1 ml 2X YT medium (17% (w/v) Bacto-tryptone (Difco Laboratories, Detroit, Michigan), 1% (w/v) Bacto-yeast extract (Difco Laboratories, Detroit, Michigan) and 0.09 M NaCl). The culture was grown overnight at 37°C with shaking, one loop streaked on a minimal media plate (pH7.4) (MMP) (0.04 M sodium phosphate dibasic (Na_2HPO_4), 0.02 M KH_2PO_4, 0.02 M NH_4Cl, 0.1 M $MgCl_2$, 0.1 M calcium chloride ($CaCl_2$), 0.1 M thiamine hydrochloride, 0.1% (w/v) glucose and 1.5% (w/v) Bacto-agar (Difco Laboratories, Detroit, Michigan)) and incubated overnight at 37°C. A single colony from the MMP plate was used to inoculate 5 ml of 2X YT media and grown overnight at 37°C. 1 ml of overnight culture was used to inoculate 100 ml of 2X YT media and grown at 37°C until reaching an optical density at 600 nanometers (OD_{600}) reading of 0.4-0.5. The cells were sedimented by centrifugation at 2,500 g for 15 min at 4°C and resuspended in 10 ml of ice-cold TSS buffer (pH 6.5) (1% (w/v) Bacto-tryptone, 0.5%

(w/v) Bacto-yeast extract, 10% polyethylene glycol (PEG, M.W. 3350), 5% (v/v) dimethylsulfoxide (DMSO) and 0.05 M $MgCl_2$). 1 ml of competent bacteria was added to 50 ul of ligation reaction and placed on ice for 45 min. A 2 min incubation at 42°C was performed to terminate the transformation.

Phage rescue of phagemid with the ScFv insert from the transformed *E. coli* TG1 was performed with the incorporation of helper phage M13K07. 900 ul of transformed cells was added to 9.1 ml of 2X YT-G media (2X YT media containing 2% glucose (w/v)) in a 15 ml polypropylene centrifuge tube and incubated at 37°C with shaking for 1 hr. 50 ul of ampicillin (2 % (w/v)) and 4 x 10^{10} plaque forming units (pfu) of M13K07 were added to the cell suspension and incubated for 1 hr at 37°C. The cells were sedimented by centrifugation at 1,000 g for 10 min, the supernatant discarded and the cells resuspended in 10 ml 2X YT-AK media (2X YT media containing 0.01% (w/v) ampicillin and 0.005% (w/v) kanamycin). The cells were incubated overnight at 37°C and centrifuged at 1,000 g for 20 min. The supernatant containing 10^{10}-10^{11} transducing units (tu) of recombinant phage/ml was transferred to a new tube and panning performed.

Selection of Antigen Positive Recombinant Phage Antibodies

Panning was carried out to select for positivity against red blood cell antigens. ABO red blood cell types A1, A2, B and two sets of O cells were utilized. 1 x 10^6 red blood cells were washed 3 times with 1 ml PBS in 1.5 ml polypropylene tubes. Each wash consisted of resuspension of cells in 1 ml of PBS, centrifugation at 500 g for 2 min and supernatant discarded. Following the final wash the cells were blocked by being incubated at RT for 1 hr in 1 ml PBS containing 3% bovine serum albumin (BSA) (w/v) (PBS-BSA), washed 3 times as previously described and resuspended to a final concentration of 1 x 10^6 cells/ml. 100 ul of each red blood cell suspension was incubated with 1.5 x 10^{10} tu of rescued phage for 30 min. A1, A2, B cells with phage were incubated at 4°C and O cells at 37°C. 100 ul of PBS-BSA was used as a negative control. The cells were washed with ice cold PBS 4 times, each wash consisting of centrifugation at 1,000 g for 10 min at 4°C followed by resuspension of cells in 10 ml ice cold PBS. 200 ul of ddHOH was added to the red cell pellets after the final wash to lyse the red blood cells and each lysate used to transform 1 ml competent *E. coli* TG1. Positive control of 200 ul of rescued phage and an additional negative control of 200 ul of ddHOH were also used. 100 ul of transformed bacteria was added to 100 ul of 2X YT-AK media and incubated at 37°C with shaking for one

hour. 100 ul of the suspension was then plated on SOBAG plates (2% (w/v) Bacto-tryptone, 0.5% (w/v) Bacto-yeast extract, 8 mM NaCl, 10 mM $MgCl_2$, 0.1 M glucose, 0.01% (w/v) ampicillin and 1.5% (w/v) Bacto-agar), incubated overnight at 37°C and observed for colonies.

Determination of Specificity

Each colony observed on SOBAG plates was used to inoculate 10 ml of 2X YT media and the culture incubated overnight at 37°C. A 1 ml glycerol stock of each phage expressed was made by mixing 0.84 ml of culture with 0.16 ml of sterile glycerol and followed by freezing at -70°C.

1 ml of frozen bacterial stock was thawed at room temperature and added to 9 ml 2X YT-AG media (2X YT media supplemented with 100ug/ul ampicillin and 2% glucose). The culture was incubated at 37°C until an OD_{600} of 0.8 was reached. $4x10^{10}$ pfu of M13K07 was added and the culture incubated for another hour at 37°C, centrifuged for 10 min at 1,000g, the supernatant discarded and the pellet resuspended in 10 ml 2X YT-AK media. The culture was incubated overnight at 37°C, centrifuged for 20 min at 1,000g and the supernatant containing phage expressed ScFv filtered through a 0.45 micron (um) filter then tested.

I) Inhibition of Agglutination

The first test performed in order to determine specificity was the inhibition of agglutination. In a 96 well plate, 50 ul of blocked red blood cells were incubated with 100 ul phage expressed ScFv for 45 min at 4°C. The plate was centrifuged at 200 g for 10 min and checked for agglutination. The plate was centrifuged again and the supernatant removed. Serial dilutions of antisera specific for the red blood cells tested were made to determine a dilution yielding a 2+ reaction. 50 ul of antisera at the 2+ reaction dilution and one dilution above and below, were added to the corresponding red blood cells. The plate was centrifuged at 200 g for 10 min and read macroscopically for agglutination.

II) Enzyme Linked Immunosorbent Assay (ELISA)

An ELISA assay was the second test performed to determine specificity. 100 ul of phage expressed ScFv was added to a 96 well polystyrene plate and incubated for 1 hr at RT. The supernatant was removed and 200 ul of blocking buffer (1% PBS-BSA) was added to the plate and incubated for 1 hr at RT. The supernatant was removed and 50 ul blocked red blood cells were added and incubated for 45 min at 4°C. The plate was centrifuged, the supernatant removed and the plate washed 4 times with 200 ul blocking buffer. 200 ul of

horseradish peroxidase (HRP)/Anti-M13 conjugate (1:5000 dilution) was added and incubated for 1 hr at RT. The plate was washed in 6X blocking buffer and 200 ul ABTS substrate (2',2'-Azino-Bis(3-Ethylbenzthiazoline-6-Sulphonic Acid) Diammonium, 2 mg/ml in 30% hydrogen peroxide) added. The plate was incubated for 20 min at RT and 150 ul of supernatant transferred to a new plate and read on an ELISA reader using EIA Reader, Model EL307 (Bio-Tek Instruments, Inc., Burlington, Vermont).

The ELISA was also performed in 1.5 ml polypropylene, conical microcentrifuge tubes using red cell membranes in place of red blood cells. The red cell membranes were prepared by lysing blocked red blood cells with 200 ul ddHOH and centrifugation at 1200g for 2 min. The membranes were washed twice with ddHOH and resuspended in PBS and the ELISA performed as described above.

III) Anti-E tag antibody

A mouse Anti-E tag antibody was screened in order to be used as a primary antibody in the ELISA assay before its utilization in an ELISA with phage expressed ScFv's bound to red blood cells. The ELISA was performed as described above in polystyrene plates, with the following modifications. Following incubation with blocking buffer, 100 ul of mouse Anti-E was added and incubated for 1 hr at RT. The wells

were washed three times with 0.05% Tween 20, and 100 ul of Anti-Mouse IgG/HRP conjugate added and incubated for 1 hr at RT. The plate was washed three times with 0.05 % Tween 20 and 200 ul of ABTS substrate added and color change observed after 20 min. A positive control of mouse IgG Anti-Human primary antibody was run as a positive control in parallel in addition to a negative control of 2X YT-AK media.

IV) Solid Phase Capture of Erythrocytes

The solid phase capture assays were run in a third attempt to determine specificity. Round and flat bottom, 96 well polystyrene plates were coated with 100 ul phage expressed ScFv and incubated for 1 hr at RT. The supernatant was removed and 50 ul blocked red blood cells added, followed by a 1 hr incubation at RT. The plate was washed with 200 ul PBS until red blood cells were observed as removed from the wells coated with 2X YT-AK as a negative control. The remaining wells were scored for presence of red blood cells observed under microscopy.

V) Secondary Panning and Western Blot Analysis

A fourth method to determine specificity was the secondary panning of phage expressed ScFv's with a Western blot analysis of the supernatants post-panning. 1 ml of phage expressed ScFv was concentrated to 100 ul volume by

fixed angle centrifugation at 100g for 30 min in centricon®-100 microconcentrator (Amicon, Danvers, Massachusetts). 100 ul of concentrated phage expressed ScFv was panned against 100 ul of blocked red blood cells and plated on SOBAG plates as previously described.

The preliminary Western blot performed was the electrophoresis of 100 ul of lysed red blood blod cells post-panning with 50 ul non-reducing sample buffer (10%(v/v) 1M Tris, pH 6.8, 3%(w/v) SDS and 16% (v/v) glycerol) on a 12% SDS-PAGE reducing gel following the methods of Laemmli (Cleveland, *et al.* 1977), and run overnight at 35 milliamps (mamps). All other Western blots were performed with the electrophoresis of 100 ul of supernatant post-panning with 50 ul of non-reducing buffer and the remaining red blood cells lysed, reinfected into competent bacteria and plated as described previously.

The proteins were transferred from the gel to Bio-Blot-NC (Costar, Corning New York) nitrocellulose paper by electrophoresis in transfer buffer (28 mM Tris, 0.21 M glycine and 22% (v/v) methanol (MeOH)) for 3 hr at 400 mamps by the methods of Towbin (Towbin, *et al.*, 1979). Following transfer, the membrane was blocked in 3% PBS-BSA with 0.5% Tween 20 for 1 hr at RT, and incubated with 10 ml HRP/Anti-M13 conjugate (1:5000) for 1 hr at RT. The membrane was washed 3 times in 0.5% PBS-BSA with 0.01% Tween 20, drained

and detection performed by chemiluminescence with 7 ml total of a mixture of equal volumes ECL™ Detection reagents 1 and 2 (Amersham Life Science, Buckinghamshire, ENGLAND) on the protein side for 60 sec. In order to detect the maximum light emission of 428 nm wavelength being produced by the chemiluminescent reaction, the membrane was drained, covered in plastic wrap and, in a dark room, exposed for 1 hr to ECL Hyperfilm™ which is a blue-light sensitive autoradiography film. The film was developed using GBX® Developer and replenisher (Kodak, Rochester, New York), fixed with GBX Fixer® and replenisher (Kodak, Rochester, New York), washed and drained at RT for 1 hr.

One round of supernatant post-panning, in addition to Western blot analysis was stained with Coomassie blue. Following gel electrophoresis, the gel was incubated in 200 ml 10% (v/v) Trichloroacetic acid (TCA), for 30 min. The TCA was discarded and 300 ml Coomassie Blue stain (10% (v/v) Acetic acid (HOAC), and 0.25% (w/v) Coomassie blue) added and incubated for 2 hr at RT. The gel was rinsed once and incubated overnight in 200 ml Destain buffer (10% (v/v) HOAC and 25% (v/v) MeOH). The destain was removed and the gel incubated in 200 ml 10% (v/v) HOAC for 1 hr. The gel was then inserted between 2 membranes and dried for 3 hr in gel dryer.

VI) Secondary Panning of Phage Expressed ScFv V_H5-$V_\lambda5$-3

50 ul Phage expressed ScFv 3 was panned against 50 ul blocked 3% human red blood cells, human white blood cells, human platelets, canine red blood cells and rat red blood cells. The panning procedure used was as previously described followed by lysis of the cells with 100 ul ddHOH, transformation of competent *E. coli* TG1 and plating on SOBAG plates. The presence or absence of colonies on the plates was observed after overnight incubation at 37°C.

RESULTS

1st strand cDNA was amplified from mRNA obtained from both human and canine. The efficiency of the synthesis was tested using human β-actin primers to detect the presence of cDNA (Figure 9).

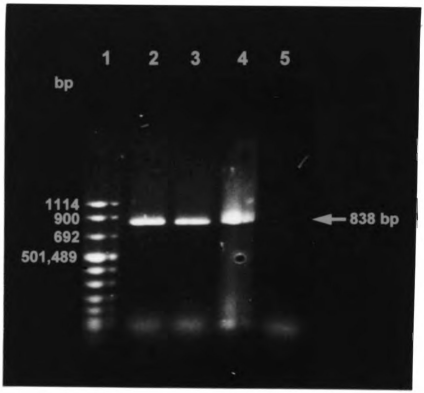

Figure 9. PCR amplification of 1st strand cDNA. Agarose gel electrophoresis of 838 bp PCR product using human β-actin primers. Lane 1 Molecular weight marker. Lane2 Canine sample. Lane 3 Human sample. Lane 4 Positive control. Lane 5 Negative control.

1st strand cDNA and genomic DNA were used as template to amplify VH and V_κ and V_λ genes using HuVHBack primers and HuJHFOR primers (Table 4). The VH and VL genes successfully amplified were used for a second round of amplification in order to generate assembled ScFv gene repertoires.

Table 4. Summary of Amplified cDNA chains.
Sizes in bp of human and canine amplified V cDNA fragments using VHBACK and JHFOR primers.

Amplified V cDNA	Human	Canine
VH1	-	-
VH2	375	-
VH3	169	155
VH4	147	89
VH5	210	153
VH6	250	-
Vκ1	185	-
Vκ2	354	276
Vκ3	-	-
Vκ4	139	170
Vκ5	281	-
Vλ1	-	-
Vλ2	422	210
Vλ3a	434	-
Vλ3b	-	-
Vλ4	400	-
Vλ5	344	-

The linker DNA was used to generate ScFv gene repertoires. The repertoires were created by the amplification of VH and VL cDNA in various combinations using the the HuVHBACK primer on the 5' end and the HuJLFOR

primer on the 3'end with the linker DNA template to join the fragments together. The successful assembly was accomplished in the combination of human V_H5 and $V_\lambda5$. The assembled V_H5-$V_\lambda5$ ScFv repertoire was reamplified with HuVH5aBACKSfi and HuJλ5FORNOT primers containing restriction sites to generate a 699 bp fragment of assembled ScFv V_H5-$V_\lambda5$ repertoire with *Not*I and *Sfi*I restriction sites (Figure 10). Additional bands seen were the result of amplification of other fragment sizes due to the presence of primers used in the previous reactions.

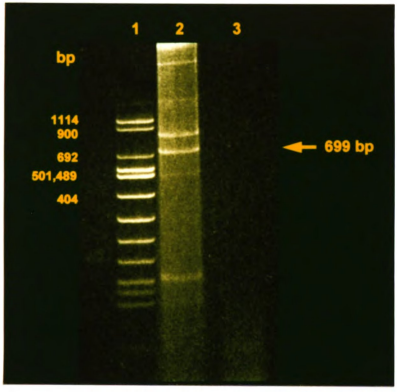

Figure 10. V_H5-$V_\lambda5$ ScFv Repertoire with Restriction Sites. Polyacrylamide gel electrophoresis of assembled 699 bp (indicated by arrow) V_H5-$V_\lambda5$ ScFv repertoire containing restriction sites *Sfi*I at the 5'end and *Not*I at the 3' end generated using primers from Table 3. Lane 1 molecular weight marker. Lane 2 Human V_H5-$V_\lambda5$ ScFv repertoire with restriction sites. Lane 3 Negative Control.

The assembled V_H5-$V_\lambda5$ repertoire with restriction sites was digested with *Not*I and *Sfi*I restriction enzymes and ligated into pCANTAB 5 E vector (Figure 11).

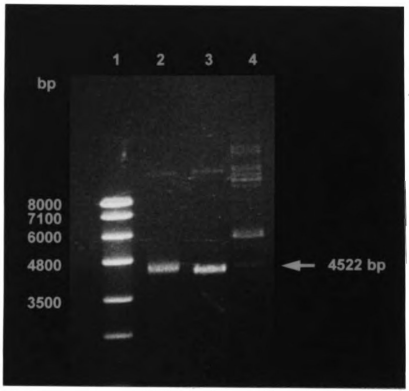

Figure 11. pCANTAB 5 E and V_H5-$V_\lambda5$ Repertoire Ligation. Agarose gel electrophoresis of 4522 bp fragment (indicated by arrow) of pCANTAB 5 E ligated with V_H5-$V_\lambda5$ repertoire after digestion with *Not*I and *Sfi*I restriction enzymes. Lane 1 Molecular weight marker. Lane 2 pCANTAB V_H5-$V_\lambda5$ repertoire. Lane 3 pCANTAB 5 E with no insert. Lane 4 pCANTAB 5 E with control ScFv insert.

The vector with V_H5-$V_\lambda5$ repertoire inserted was used to infect competent *E. coli* TG1 and the bacteria grown in culture. M13K07 helper phage was added to the culture and phage expressed V_H5-$V_\lambda5$ produced. The phage were panned against red blood cells for selection and the cells washed. The red blood cells were lysed, the phage bound to the cell eluted and used to reinfect competent *E. coli* TG1. After selection with ampicillin kanamycin media, 5 colonies on SOBAG plates were observed after two rounds of selection; 3 colonies were observed on plates containing bacteria infected with phage after panning against B cells, the second round of panning produced 2 colonies against A1 cells. The first three colonies were designated V_H5-$V_\lambda5$-1, 2 and 3, the third and fourth colonies, V_H5-$V_\lambda5$-4 and 5 (Figure 12).

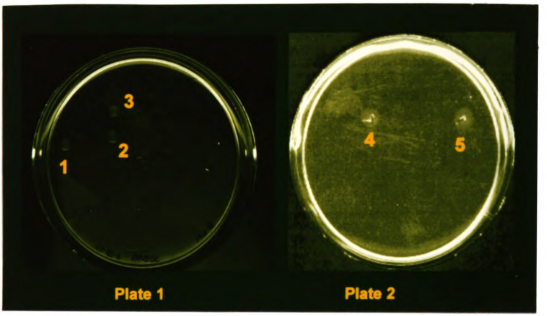

Figure 12. Growth of Colonies on SOBAG Plates.
Colonies of *E coli* TG1 infected with phage expresses V_H5-$V_\lambda5$ grown on SOBAG plates after panning against red cells and eluted. Plate 1 V_H5-$V_\lambda5$ 1, 2 and 3 from phage expressed V_H5-$V_\lambda5$ eluted from Type B red blood cells. Plate 2 V_H5-$V_\lambda5$ 1, 2 and 3 from phage expressed V_H5-$V_\lambda5$ eluted from Type A1 red blood cells.

The results of phage expressed V_H5-$V_\lambda5$ 1, 2 and 3 screened for specificity by inhibition of agglutination using A1, A2, B and two sets of O cells, SCI and SCII are seen in Table 5.

Table 5. Results of Inhibition of Agglutination Test. Phage expressed V_H5-$V_\lambda5$ 1, 2, and 3 incubated with red blood cells followed by the addition of control antisera to observe inhibition of agglutination. + indicates the observation of agglutination.

	A cells + αA1			B cells + αB			SCI + αD			SCII + αD		
	1:1	1:2	1:4	1:8	1:16	1:32	1:1	1:2	1:4	1:2	1:4	1:8
V_H5-$V_\lambda5$ 1	+	+	+	+	+	+	+	+	+	+	+	+
V_H5-$V_\lambda5$ 2	+	+	+	+	+	+	+	+	+	+	+	+
V_H5-$V_\lambda5$ 3	+	+	+	+	+	+	+	+	+	+	+	+
2X YT-AK	+	+	+	+	+	+	+	+	+	+	+	+

Phage expressed V_H5-$V_\lambda5$ 1, 2, and 3 were tested in triplicate by an ELISA assay to detect specificity using HRP/Anti-M13 conjugate (Table 6).

Table 6. ELISA of Phage Expressed V_H5-$V_\lambda5$ 1, 2, and 3. Results of phage expressed V_H5-$V_\lambda5$ 1, 2, and 3 incubated with red blood cells A1, A2, B and O cells were detected with HRP/Anti-M13 conjugate and 1X ABTS substrate. Positive control wells included no incubation with any cell types.

RBC	V_H5-$V_\lambda5$ 1			V_H5-$V_\lambda5$ 2			V_H5-$V_\lambda5$ 3			2X YT-AK		
A1	0.26	0.20	0.41	0.29	0.24	0.31	0.33	0.27	0.46	0.47	0.51	0.47
A2	0.02	0.01	0.10	0.09	0.14	0.10	0.1	-0.0	0.04	-0.0	-0.1	0.07
B	-0.0	-0.0	-0.1	-0.0	-0.0	0.07	-0.0	0.16	0.03	-0.0	0.11	0.13
SC I	0.23	0.20	0.09	0.06	0.20	0.11	0.09	0.23	0.14	0.08	0.18	0.13
SC II	0.01	0.10	0.06	0.12	0.02	0.02	0.02	0.05	0.16	0.07	0.12	0.03
No Cells	0.01	-0.0		-0.0	-0.0		-0.0	-0.0		-0.0	-0.0	
+ Ctrl	0.24	0.22										

Phage expressed V_H5-$V_\lambda5$ 1, 2, 3, 4 and 5 were used for a secondary round of panning against A1, A2, B and O red blood cells. The red blood cells were lysed and used to infect competent *E. coli* TG1 and grown on SOBAG plates after selection with 2X YT-AK media (Table 7).

Table 7. Summary of secondary panning results. Numbers of colonies observed on SOBAG plates after 4 rounds of phage expressed V_H5-$V_\lambda5$ 1, 2, 3, 4 and 5 secondary panning against A1, A2, B and O cells, eluted and used to reinfect competent *E. coli* TG1.

	A1 Cells	A2 Cells	B Cells	O Cells	No Cells
V_H5-$V_\lambda5$ **1**					
Round 1	>20	0	0	0	0
Round 2	>20	>20	>20	>20	0
Round 3	0	0	0	0	0
Round 4	>20	>20	>20	>20	0
V_H5-$V_\lambda5$ **2**					
Round 1	0	0	0	0	0
Round 2	>20	4	5	>20	0
Round 3	>20	>20	>20	>20	0
Round 4	>20	>20	>20	>20	0
V_H5-$V_\lambda5$ **3**					
Round 1	0	0	0	1	0
Round 2	>20	>20	>20	>20	0
Round 3	>20	>20	>20	>20	0
Round 4	>20	>20	>20	>20	0
V_H5-$V_\lambda5$ **4**					
Round 1	0	0	0	0	0
Round 2	0	0	0	0	0
Round 3	0	0	0	0	0
Round 4	0	0	0	0	0
V_H5-$V_\lambda5$ **5**					
Round 1	3	>20	>20	2	0
Round 2	>20	>20	>20	>20	0
Round 3	>20	>20	>20	>20	0
Round 4	0	>20	>20	0	0

Following panning against red blood cells, the supernatants of V_H5-$V_\lambda5$ 1-5 were used in Western blot analysis. The results of the analysis for V_H5-$V_\lambda5$ 1 are shown in Figure 13; V_H5-$V_\lambda5$ 2 in Figure 14; V_H5-$V_\lambda5$ 3 in Figure 15; V_H5-$V_\lambda5$ 4 in Figure 16 and V_H5-$V_\lambda5$ 5 in Figure 17. The bands observed in the gels and their corresponding sizes were also summarized (Table 8).

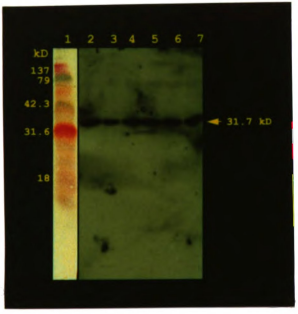

Figure 13. Western Blot Analysis V_H5-$V_\lambda5$-1.
31.7 kD bands (indicated by arrow) of supernatants of phage expressed V_H5-$V_\lambda5$-1 post-panning against red blood cells A1, A2, B and O (See Table 8).
Lane 1 Molecular weight marker. Lane 2 Supernatant of phage expressed V_H5-$V_\lambda5$-1 post-panning against A1 red blood cells. Lane 3 Supernatant of phage expressed V_H5-$V_\lambda5$-1 post-panning against A2 red blood cells. Lane 4 Supernatant of phage expressed V_H5-$V_\lambda5$-1 post-panning against B red blood cells. Lane 5 Supernatant of phage expressed V_H5-$V_\lambda5$-1 post-panning against O red blood cells Lane 6 Supernatant of phage expressed V_H5-$V_\lambda5$-1 not panned against red cells. Lane 7 Supernatant of phage expressed vector containing no ScFv insert.

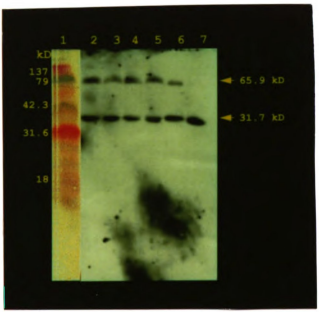

Figure 14. Western Blot Analysis V_H5-$V_\lambda5$-2.
31.7 and 65.9 kD bands (indicated by arrows) of supernatants of phage expressed V_H5-$V_\lambda5$-2 post-panning against red blood cells A1, A2, B and O (See Table 8).
Lane 1 Molecular weight marker. Lane 2 Supernatant of phage expressed V_H5-$V_\lambda5$-2 post-panning against A1 red blood cells. Lane 3 Supernatant of phage expressed V_H5-$V_\lambda5$-2 post-panning against A2 red blood cells. Lane 4 Supernatant of phage expressed V_H5-$V_\lambda5$-2 post-panning against B red blood cells. Lane 5 Supernatant of phage expressed V_H5-$V_\lambda5$-2 post-panning against O red blood cells. Lane 6 Supernatant of phage expressed V_H5-$V_\lambda5$-2 not panned against red cells. Lane 7 Supernatant of phage expressed vector containing no ScFv insert.

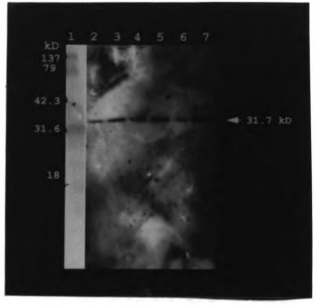

Figure 15. Western Blot Analysis V_H5-$V_\lambda5$-3.
31.7 kD bands (indicated by arrow) of supernatants of phage expressed V_H5-$V_\lambda5$-3 post-panning against red blood cells A1, A2, B and O (See Table 8).
Lane 1 Molecular weight marker. Lane 2 Supernatant of phage expressed V_H5-$V_\lambda5$-3 post-panning against A1 red blood cells. Lane 3 Supernatant of phage expressed V_H5-$V_\lambda5$-3 post-panning against A2 red blood cells. Lane 4 Supernatant of phage expressed V_H5-$V_\lambda5$-3 post-panning against B red blood cells. Lane 5 Supernatant of phage expressed V_H5-$V_\lambda5$-3 post-panning against O red blood cells. Lane 6 Supernatant of phage expressed V_H5-$V_\lambda5$-3 not panned against red cells. Lane 7 Supernatant of phage expressed vector containing no ScFv insert.

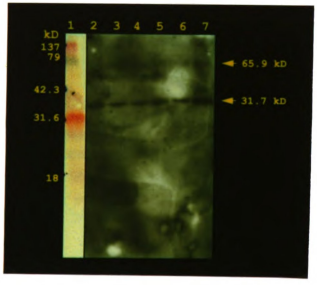

Figure 16. Western Blot Analysis V_H5-$V_\lambda5$-4.
31.7 and 65.9 kD bands (indicated by arrows) of supernatants of phage expressed V_H5-$V_\lambda5$-4 post-panning against red blood cells A1, A2, B and O (See Table 8).
Lane 1 Molecular weight marker. Lane 2 Supernatant of phage expressed V_H5-$V_\lambda5$-4 post-panning against A1 red blood cells. Lane 3 Supernatant of phage expressed V_H5-$V_\lambda5$-4 post-panning against A2 red blood cells. Lane 4 Supernatant of phage expressed V_H5-$V_\lambda5$-4 post-panning against B red blood cells. Lane 5 Supernatant of phage expressed V_H5-$V_\lambda5$-4 post-panning against O red blood cells. Lane 6 Supernatant of phage expressed V_H5-$V_\lambda5$-4 not panned against red cells. Lane 7 Supernatant of phage expressed vector containing no ScFv insert.

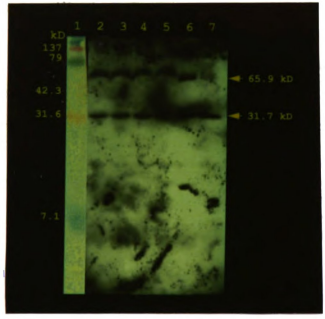

Figure 17. Western Blot Analysis V_H5-$V_\lambda5$-5.
31.7 and 65.9 kD bands (indicated by arrows) of supernatants of phage expressed V_H5-$V_\lambda5$-5 post-panning against red blood cells A1, A2, B and O (See Table 8).
Lane 1 Molecular weight marker. Lane 2 Supernatant of phage expressed V_H5-$V_\lambda5$-5 post-panning against A1 red blood cells. Lane 3 Supernatant of phage expressed V_H5-$V_\lambda5$-5 post-panning against A2 red blood cells. Lane 4 Supernatant of phage expressed V_H5-$V_\lambda5$-5 post-panning against B red blood cells. Lane 5 Supernatant of phage expressed V_H5-$V_\lambda5$-5 post-panning against O red blood cells. Lane 6 Supernatant of phage expressed V_H5-$V_\lambda5$-5 not panned against red cells. Lane 7 Supernatant of phage expressed vector containing no ScFv insert.

Table 8. Summary of Band Sizes (kD)- Western Blot Analysis
Fragments detected by Western Blot Analysis of supernatants of V_H5-$V_\lambda5$ 1-5 post panning against red blood cells A1, A2, B and O (Figures 13-17).
Positive control indicates supernatants not panned against red cells. No insert indicates supernatant of phage expressed vector with no ScFv insert.

	A1	A2	B	O	Pos Control	No Insert
V_H5-$V_\lambda5$ 1	31.7	31.7	31.7	31.7	31.7	31.7
V_H5-$V_\lambda5$ 2	65.9 31.7	65.9 31.7	65.9 31.7	65.9 31.7	65.9 31.7	31.7
V_H5-$V_\lambda5$ 3	31.7	31.7	31.7	31.7	31.7	31.7
V_H5-$V_\lambda5$ 4	31.7	31.7	31.7	31.7	31.7	31.7 65.9
V_H5-$V_\lambda5$ 5	65.9 31.7	65.9 31.7	65.9 31.7	65.9 31.7	65.9 31.7	31.7

Coomassie Blue Staining of the supernatants and the results for V_H5-$V_\lambda5$ 1 are shown in Figure 18; V_H5-$V_\lambda5$ 2 in Figure 19; V_H5-$V_\lambda5$ 3 in Figure 20; V_H5-$V_\lambda5$ 4 in Figure 21 and V_H5-$V_\lambda5$ 5 in Figure 22. The bands observed in the gels and their corresponding sizes were summarized in Table 9.

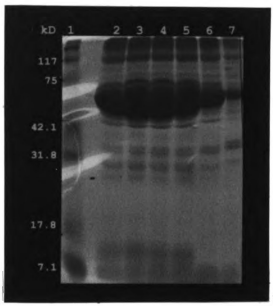

Figure 18. Coomassie Blue Staining of V_H5-$V_\lambda5$-1. Supernatants of phage expressed V_H5-$V_\lambda5$-1 post-panning against red blood cells A1, A2, B and O (See Table 9). Lane 1 Molecular weight marker. Lane 2 Supernatant of phage expressed V_H5-$V_\lambda5$-1 post-panning against A1 red blood cells. Lane 3 Supernatant of phage expressed V_H5-$V_\lambda5$-1 post-panning against A2 red blood cells. Lane 4 Supernatant of phage expressed V_H5-$V_\lambda5$-1 post-panning against B red blood cells. Lane 5 Supernatant of phage expressed V_H5-$V_\lambda5$-1 post-panning against O red blood cells Lane 6 Supernatant of phage expressed V_H5-$V_\lambda5$-1 not panned against red cells. Lane 7 Supernatant of phage expressed vector containing no ScFv insert.

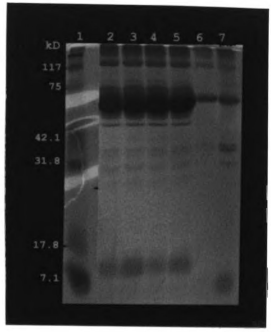

Figure 19. Coomassie Blue Staining of V_H5-$V_\lambda5$-2. Supernatants of phage expressed V_H5-$V_\lambda5$-2 post-panning against red blood cells A1, A2, B and O (See Table 9). Lane 1 Molecular weight marker. Lane 2 Supernatant of phage expressed V_H5-$V_\lambda5$-1 post-panning against A1 red blood cells. Lane 3 Supernatant of phage expressed V_H5-$V_\lambda5$-1 post-panning against A2 red blood cells. Lane 4 Supernatant of phage expressed V_H5-$V_\lambda5$-1 post-panning against B red blood cells. Lane 5 Supernatant of phage expressed V_H5-$V_\lambda5$-1 post-panning against O red blood cells Lane 6 Supernatant of phage expressed V_H5-$V_\lambda5$-1 not panned against red cells. Lane 7 Supernatant of phage expressed vector containing no ScFv insert.

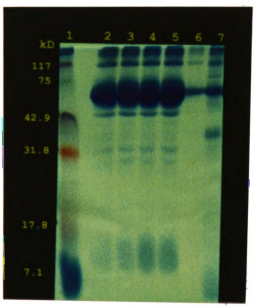

Figure 20. Coomassie Blue Staining of V_H5-$V_\lambda5$-3. Supernatants of phage expressed V_H5-$V_\lambda5$-3 post-panning against red blood cells A1, A2, B and O (See Table 9). Lane 1 Molecular weight marker. Lane 2 Supernatant of phage expressed V_H5-$V_\lambda5$-1 post-panning against A1 red blood cells. Lane 3 Supernatant of phage expressed V_H5-$V_\lambda5$-1 post-panning against A2 red blood cells. Lane 4 Supernatant of phage expressed V_H5-$V_\lambda5$-1 post-panning against B red blood cells. Lane 5 Supernatant of phage expressed V_H5-$V_\lambda5$-1 post-panning against O red blood cells Lane 6 Supernatant of phage expressed V_H5-$V_\lambda5$-1 not panned against red cells. Lane 7 Supernatant of phage expressed vector containing no ScFv insert.

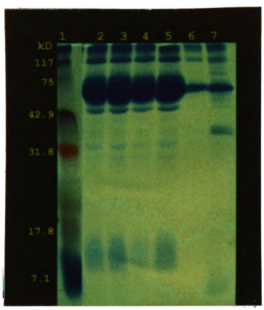

Figure 21. Coomassie Blue Staining of V_H5-$V_\lambda5$-4. Supernatants of phage expressed V_H5-$V_\lambda5$-4 post-panning against red blood cells A1, A2, B and O (See Table 9). Lane 1 Molecular weight marker. Lane 2 Supernatant of phage expressed V_H5-$V_\lambda5$-1 post-panning against A1 red blood cells. Lane 3 Supernatant of phage expressed V_H5-$V_\lambda5$-1 post-panning against A2 red blood cells. Lane 4 Supernatant of phage expressed V_H5-$V_\lambda5$-1 post-panning against B red blood cells. Lane 5 Supernatant of phage expressed V_H5-$V_\lambda5$-1 post-panning against O red blood cells Lane 6 Supernatant of phage expressed V_H5-$V_\lambda5$-1 not panned against red cells. Lane 7 Supernatant of phage expressed vector containing no ScFv insert.

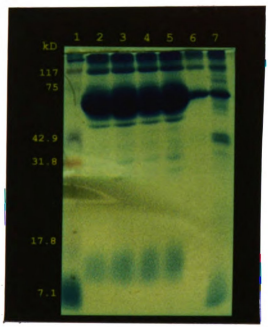

Figure 22. Coomassie Blue Staining of V_H5-$V_\lambda5$-5. Supernatants of phage expressed V_H5-$V_\lambda5$-5 post-panning against red blood cells A1, A2, B and O (See Table 9). Lane 1 Molecular weight marker. Lane 2 Supernatant of phage expressed V_H5-$V_\lambda5$-1 post-panning against A1 red blood cells. Lane 3 Supernatant of phage expressed V_H5-$V_\lambda5$-1 post-panning against A2 red blood cells. Lane 4 Supernatant of phage expressed V_H5-$V_\lambda5$-1 post-panning against B red blood cells. Lane 5 Supernatant of phage expressed V_H5-$V_\lambda5$-1 post-panning against O red blood cells Lane 6 Supernatant of phage expressed V_H5-$V_\lambda5$-1 not panned against red cells. Lane 7 Supernatant of phage expressed vector containing no ScFv insert.

Table 9. Summary of Band Sizes (kD)- Coomassie Blue Stain. Fragments detected by Coomassie Blue Staining of supernatants of V_H5-$V_\lambda5$ 1-5 post panning against red blood cells A1, A2, B and O (Figures 18-22).
Positive control indicates supernatants not panned against red cells. No insert indicates supernatant of phage expressed vector with no ScFv insert.

	A1	A2	B	O	Pos Control	No Insert
V_H5-$V_\lambda5$ 1	123.1 100.3 61.2 28.3 17.1 15.1 8.5	123.1 100.3 61.2 28.3 17.1 15.1 8.5	123.1 100.3 61.2 28.3 17.1 15.1 8.5	123.1 100.3 61.2 28.3 17.1 15.1 8.5	123.1 100.3 61.2 28.3 17.1 15.1 8.5	123.1 100.3 61.2 39.4 5.1
V_H5-$V_\lambda5$ 2	123.1 100.3 61.2 28.3 17.1 15.1 8.5	123.1 100.3 61.2 28.3 17.1 15.1 8.5	123.1 100.3 61.2 28.3 17.1 15.1 8.5	123.1 100.3 61.2 28.3 17.1 15.1 8.5	123.1 100.3 61.2 28.3 17.1 15.1 8.5	123.1 100.3 61.2 39.4 5.1
V_H5-$V_\lambda5$ 3	123.1 100.3 61.2 28.3 17.1 15.1 8.5	123.1 100.3 61.2 28.3 17.1 15.1 8.5	123.1 100.3 61.2 28.3 17.1 15.1 8.5	123.1 100.3 61.2 28.3 17.1 15.1 8.5	123.1 100.3 61.2 28.3 17.1 15.1 8.5	123.1 100.3 61.2 39.4 5.1
V_H5-$V_\lambda5$ 4	123.1 100.3 61.2 28.3 17.1 15.1 8.5	123.1 100.3 61.2 28.3 17.1 15.1 8.5	123.1 100.3 61.2 28.3 17.1 15.1 8.5	123.1 100.3 61.2 28.3 17.1 15.1 8.5	123.1 100.3 61.2 28.3 17.1 15.1 8.5	123.1 100.3 61.2 39.4 5.1
V_H5-$V_\lambda5$ 5	123.1 100.3 61.2 28.3 17.1 15.1 8.5	123.1 100.3 61.2 28.3 17.1 15.1 8.5	123.1 100.3 61.2 28.3 17.1 15.1 8.5	123.1 100.3 61.2 28.3 17.1 15.1 8.5	123.1 100.3 61.2 28.3 17.1 15.1 8.5	123.1 100.3 61.2 39.4 5.1

On separate days in duplicate, phage expressed V_H5-$V_\lambda5$ 3 was used to pan against pooled human red blood cells, human white blood cells. human platelets, canine red blood cells and rat red blood cells. The bound phage was eluted and used to infect competent *E. coli* TG1. Infected bacteria were selected for with 2X YT-AK media and plated on SOBAG plates. Colonies were observed after overnight incubation. The results of the panning are shown in Table 10.

Table 10. Secondary Panning Results of V_H5-$V_\lambda5$ 3.
V_H5-$V_\lambda5$ 3 panned against human red blood cells, human white blood cells, human platelets, canine red blood cells and rat red blood cells. Number of colonies observed on SOBAG plates of *E. coli* TG1 infected with phage expressed V_H5-$V_\lambda5$ 3 eluted post-panning against human rat and canine cells.

Cell Type	**Day 1**		**Day 2**	
	Round 1	Round 2	Round 1	Round 2
Pooled Human Red Cells	9	8	0	0
Human White Blood Cells	8	>20	0	0
Human Platelets	3	21	0	0
Canine Red Blood Cells	3	9	1	0
Rat Red Blood Cells	>20	>20	2	0
Negative Control	0	0	0	0
E. coli TG1 Non Infected	0	0	0	0
Positive Control	+	+	+	+

DISCUSSION

The successful amplification of cDNA using human β-actin primers demonstrated the isolation of RNA and subsequent cDNA synthesis to have been an efficient process (Figure 9). The amplification of V_H, V_λ, and V_κ chains from cDNA in both humans and dogs were partially successful (Table 4). The inability to amplify all chains in the human could be attributed to the lack of appropriate conditions. The number of variable genes also existed in different quantities, therefore, those existing in small quantities may require more precise parameters for amplification. In the dog, amplification of variable chains did occur, however the size of bands produced in comparison to the corresponding human chain were different. Kehoe and Capra (1972) have demonstrated homology between the species V_H regions. Specifically, the canine V_H gene sequences have been found to be homologous to the human V_H-III family . In addition, Kehoe (1982) also showed the similarities between the V_κ regions of the two species. Not much information has been published on the comparison of the V_λ regions. In humans it has been found that significant sequence diversity exists in the hypervariable loops of all variable chains, though conformations of binding sites are unaltered which results in differing number of residues in these regions

(Winter, *et al.*, 1994). Therefore, the possibility exists for differing size fragments to be amplified.

ScFv repertoire assembly was successful only in the human V_H5-$V_\lambda5$ (Figure 10). The limited quantity of PCR products amplified provided insufficient yields to allow for their purification without substantial loss of product. As a result, the amplified product was used without purification and the presence of primers from previous PCR reactions may have inhibited the assembly of other repertoires in both humans and dogs. The presence of primers used in previous PCR reactions compete for polymerase and dNTP's to further produce previously amplified products rather than the newly desired product being amplified. The purification of the product regardless of consequent loss of product may have resulted in more successful assembly.

Restriction sites were successfully generated on the V_H5-$V_\lambda5$ repertoire by digestion with *Not*I and *Sfi*I restriction enzymes which resulted in the insertion of the repertoire in the pCANTAB 5 E vector at position 2314 (Figure 11). Infection of competent *E. coli* TG1 followed by the incorporation of helper phage M13K07 produced a phage expressed ScFv V_H5-$V_\lambda5$ which was demonstrated by the growth of colonies on SOBAG plates after selection by ampicillin and kanamycin resistance.

Two rounds of panning of the phage expressed ScFv V_H5-$V_\lambda5$ against human red blood cells Type A1, A2, B and O yielded different results. One round produced three colonies against B cells and the second, two colonies against A1 cells (Figure 12). The selection of the five colonies may have been a chance event of these phage expressed ScFv's to bind antigens on the cell membranes rather than specific to the A1 and B antigens. The lack of affinity maturation in this process does not result in the production of a higher affinity ScFv. However, successive rounds lead to the production of enriched numbers of phage expressed ScFv selected for during consecutive pannings. As a result, successive rounds of panning should be more indicative of specificity.

The four rounds of secondary panning produced conflicting results of positivity and negativity. This may be partly explained by their selection efficiency dependent upon their dissociation kinetics during washing. Consistency in washing techniques and numbers of washes used play an important part in the dissociation kinetics. Experiments adjusting these factors to favor selection of higher affinity ScFv's in primary panning rounds could be used to establish a better protocol.

A second factor which plays a significant part in the selection of bound phage is the ability of multiple ScFv

fragments on a single phage to simultaneously bind the antigen (Clackson, *et al.* 1991). The inability of all ScFv's to simultaneously bind to antigen at all times may also account for inconsistent results.

Though the inhibition of agglutination experiment yielded negative results (Table 5), the concentration of phage expressed ScFv V_H5-$V_\lambda5$ to antigen was unknown. The specificity of phage expressed ScFv V_H5-$V_\lambda5$ was also unknown. Therefore the ability of corresponding antisera to agglutinate the red blood cells could be due to the specificity of phage expressed ScFv V_H5-$V_\lambda5$ to be something other than A1, A2, B and D. A second hypothesis is that the existence of phage expressed ScFv V_H5-$V_\lambda5$ may have been in inappropriate concentrations to block all the binding sites of the control antisera and not effectively inhibit agglutination.

The results of the ELISA assays were invalid due to the lysis of red blood cells by 1X ABTS substrate and subsequent release of hydrogen peroxidase from the cells causing false positives (Table 6). As a result this method of specificity determination was no longer pursued.

The solid phase erythrocyte capture assay yielded conflicting results which could also not be confirmed. Once again, the dissociation kinetics could explain the discrepancy in results, however, the subjective manner of

reporting provided an unreliable means of obtaining the results seen.

The Western blot analysis of V_H5-$V_\lambda5$ 1 (Figure 13) showed no evidence of an insert between the positive control and the phage expressed ScFv with no insert as summarized in Table 8. Coomassie Blue staining also yielded the same results as seen in Figure 18 and Table 9.

Analysis of V_H5-$V_\lambda5$ 2 showed slightly different results than V_H5-$V_\lambda5$ 1. Western blot analysis (Figure 14) show the presence of a band at 65.9 kD not seen in the "No Insert" lane (Table 8). Though the bands in lanes 2-5 seem to be a different size than seen in lane 6 they are the same band. The observance of a heavy 61.2 kD band in the Coomassie Blue stained lanes of supernatants panned against red cells (Figure 19) indicate the difference in sizes of bands to result in the shift of the 65.9 kD bands seen in the Western blot.

Western blot analysis of V_H5-$V_\lambda5$ 3 (Figure 15 and Table 8) and Coomassie Blue staining (Figure 20 and Table 9) yielded the same results as V_H5-$V_\lambda5$ 1. Western blot analysis of V_H5-$V_\lambda5$ 4 (Figure 16 and Table 8) indicate the presence of a band in the "No insert" lane at 65.9 kD which is not present in the other lanes. Coomassie Blue staining (Figure

21 and Table 9) did not duplicate the results of the presence of this band.

The analysis of V_H5-$V_\lambda5$ 5 is similar to that of V_H5-$V_\lambda5$ 2. The presence of a 65.9 kD band is seen in all lanes but the "No Insert" lane in Western blot analysis (Figure 17 and Table 8). These findings are not duplicated by Coomassie Blue staining (Figure 22 and Table 9) but the presence of a heavy 61.2 kD band is the likely explanation of the shift in band sizes seen in the Western blot between the lanes panned against red blood cells and the positive control as was also depicted in V_H5-$V_\lambda5$ 2.

The Western blot analysis (Figures 13-17 and Table 8)and Coomassie blue staining (Figures 18-22 and Table 9) techniques failed to document the existence of a band of decreased intensity upon panning in comparison to the positive control and supernatants of phage expressed ScFv V_H5-$V_\lambda5$ post-panning to indirectly indicate specificity of the phage expressed ScFv. It also provided no evidence of an insert between the positive control and the phage expressed ScFv with no insert. Though supernatants of phage expressed V_H5-$V_\lambda5$ 2 and 5 have a 65.9 kD band not detected in the "No Insert" lane, the band is present in the V_H5-$V_\lambda5$ 4 "No Insert" lane in the Western blot analysis and not in the other lanes. In secondary panning experiments V_H5-$V_\lambda1$ and 3

also exhibit positive growth indicative of the selection of a phage expressed ScFv, yet the presence of this 65.9 kD band is not detected. This band is therefore not thought to be a significant indicator of the ScFv insert.

Though Coomassie Blue is not as sensitive as the Western blot, it did show a significant band at 61.2 kD in all lanes panned against red blood cells in all phage expressed ScFv supernatants. This band has also not been considered an indicator of specificity with regard to red blood cells as it is seen in the V_H5-$V_\lambda5$ 4 gel (Figure 21) which has demonstrated negative results in all rounds of secondary panning (Table 7).

The screening of phage expressed ScFv V_H5-$V_\lambda5$-3 (Table 10) against other cell types than human red blood cells resulted from the propensity of the ScFv to bind to all red blood cells which indicated a specificity toward a membrane protein. The results of the first screening show that this could be correct. The results of the second screening do not confirm this. As discussed earlier, washing may contribute to the discrepancy in these results.

The specificity of the phage expressed ScFv V_H5-$V_\lambda5$ selected has not been determined. Due to the positive selection with ampicillin and kanamycin and the demonstration of insertion being evident, it can be concluded that there is a phage expressed ScFv.

RECOMMENDATIONS

The following recommendations have been made to improve the outcome of this project based on the results presented.

1. Purification of amplified PCR product prior to ScFv repertoire assembly may provide better template for successful amplification.
2. The use of Anti-E tag antibody in Western blots could be more sensitive and specific for analyzing results. It would also provide screening of soluble phage expressed ScFv's.
3. The further screening of the phage expressed ScFv V_H5-$V_\lambda5$ could be continued to determine its specificity using isolated membrane proteins common to all cell types.
4. The publication of canine variable gene sequences would be helpful as it could result in the design of more specific primers for the variable chains.
5. The efficiency of producing a desired phage expressed ScFv may be enhanced by the initial incubation of isolated B cells with the antigen of choice. B cells bound could then be eluted and the process of phage expression begun with a source of desired antibody genes.

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