Discussion
DISCUSSION

Defects in the immune system can have serious pathological consequences. For example, aberrant recognition of self-molecules can result in autoimmune responses. It is now believed that autoimmunity per se may not be very infrequent; however, when autoimmune T cells and auto-antibodies secreted by B cells adversely organ function, autoimmune diseases occur.

SLE is a prototypic systemic autoimmune disease. As the name suggests, multiple organs may become targets of the autoimmune response. Diagnosis is based on presence of four out of eleven clinical criteria (enumerated by the American College of Rheumatology), either simultaneously or during any period of observation. Most organ damage in SLE is believed to be antibody-mediated, though T cells play a critical role in the generation of auto-antibody responses. Auto-antibodies against some antigens can be observed as early as nine years before the onset of clinical symptoms. Although auto-antibodies against more than a hundred auto-antigens have been described, most may be epi-phenomena, and may not necessarily affect end-organ function. A few auto-immune specificities have been found to be associated with specific pathologies, however; anti-dsDNA antibodies have been linked to chronic renal failure, anti-Ro and anti-La antibodies with neonatal lupus, antibodies to the ribosomal P protein with the neuropsychiatric manifestation often seen in lupus, and anti-phospholipid (targeting cardiolipin, phosphatidylserine and other lipids) and anti-RBC antibodies (against Rh antigens, Band 3 and other antigens) with the lysis of RBC. As a result of autoimmune hemolysis, and also of non-autoimmune hemolysis in diseases such as malaria and leishmaniasis, Hb is released into circulation. Hb scavenging systems, such as haptoglobin (the Hb-binding protein) and hemopexin (the heme-binding protein) may be quickly overwhelmed. Free heme and/or released iron can exert a variety of toxic effects.

Some evidence of aberrant T cell reactivity to the Hb does exist. The NZB animals develop spontaneous auto-antibodies to their own RBCs by 6 months of age, leading to autoimmune hemolytic anemia. When autologous RBC lysate was added to splenocytes, heightened proliferative responses were observed. Incubation of the splenocytes from these animals with the autologous purified Hb resulted in the preferential proliferation of CD4+ T cells; unlike in the non-
autoimmune strain BALB/c animals, Hb-induced proliferation could not be inhibited by Hp221. Anti-Hb reactivity was also apparent in a human patient who had undergone multiple transfusions220. Using microarray analysis of auto-antibodies found in cord blood, it was found that both maternal and fetal auto-antibodies recognized alpha and beta subunits of Hb238. Such observations indicated that Hb can indeed act as auto-antigen, and such reactivity may manifest in individuals predisposed towards autoimmunity.

Auto-reactivity to other heme-containing proteins has also been observed. Grave’s Disease and Hashimoto’s Thyroiditis are organ-specific autoimmune diseases in which the thyroid gland is the target of auto-reactive antibodies and T cells. Auto-antibodies (mainly of the IgG isotype, but also IgA and IgE) and auto-reactive T cells target thyroid peroxidase (TPO)239, a membrane bound heme-containing protein involved in the synthesis of thyroid hormones240.

Lupus is characterized by aberrant apoptosis; excessive apoptosis has been observed in peripheral cells, as have defects in the uptake of apoptotic cells71,72,73,74. RN86, a human monoclonal which specifically targets apoptotic cells (Figures 1a, 1b) was generated from B cells derived from an SLE patient67. Further analysis revealed that, upon Western blots on RBC lysate, the antibody bound to a protein of molecular mass of around 64 KDa (Figure 1c). As more than 90% protein of RBCs is Hb222,224, which in its quaternary conformation is of equivalent molecular mass, ELISA on commercial hHb was performed; strong reactivity of RN86 to hHb was demonstrable. This study aimed to explore the extent, characteristics and implications of Hb auto-reactivity.

For further analyses, sera were obtained from lupus patients visiting the rheumatology clinic at the All India Institute of Medical Sciences, New Delhi. Only patients in the midst of a disease “flare” (as defined by clinical criteria such as the SLEDAI) were chosen, in the hope of obtaining sera expressing auto-antibody titres. Sera were also obtained from equivalent number of healthy individuals, to be employed as negative controls. Western blots and ELISA on human Hb demonstrated the presence of anti-hHb antibodies in a certain percentage of lupus patients (Figure 2). Control sera did not demonstrate the presence of such antibodies, revealing reactivity to be related to the on-going disease process.
A panel of SLE Sera was obtained from the Center for Disease Control and Prevention (CDC) at Atlanta. These sera were previously characterized for the presence of different auto-antibodies. Interestingly, only sera designated to contain antibodies to the Smith (Sm) antigen demonstrated the additional presence of antibodies reactive towards hHb, both on ELISA and on Western blot analysis (Figure 3); different batches of sera obtained from the CDC over a three-year period demonstrated the same pattern of reactivity, further strengthening the authenticity and validity of these observations. Though present in only about 30% of SLE patients, anti-Sm antibodies are specific to the disease and their presence is therefore included as a diagnostic criteria\(^\text{241,242}\). Unexplained associations between different auto-antibody reactivities have been reported; for example, investigators have found an association between anti-Sm reactivity and reactivity to the ribosomal P protein\(^\text{30}\). To assess if observation of the association between anti-Sm and anti-hHb auto-reactivity could be further extended, simultaneous ELISAs were conducted upon sera from Indian SLE patients, using Sm and Hb as targets (Figure 4a, b). Of the three patients demonstrating anti-hHb antibodies in their sera, two harbored anti-Sm antibodies as well; control sera did not contain significant titres of antibodies to either molecule. These observations indicated that Sm reactivity could be a sufficient but not a necessary criterion for anti-hHb reactivity. Most auto-antibodies that target Sm have been shown to be of the IgG isotype\(^\text{231,243}\). The present analysis indicated that anti-hHb reactivity could be mediated by both IgG and IgM isotypes, with the relative dominance of the isotypes varying between patients (Figure 4c).

To see if anti-hHb reactivity was a phenomenon that extended beyond classical autoimmune disease, sera obtained from patients of malaria (both *Plasmodium vivax* and *Plasmodium falciparum*) and leishmaniasis were analyzed; these diseases, like lupus, are characterized by extensive hemolysis\(^\text{244-246}\). In addition, enhanced apoptosis is observed\(^\text{225}\) and the presence of auto-reactive antibodies has been demonstrated\(^\text{247,248}\). Interestingly, a significant number of patients harbored antibodies reactive to hHb in serum (Figure 5). As indicated above, antibodies to Sm are conventionally believed to occur only in lupus. Some reports, however, have demonstrated the presence of such antibodies in patients suffering from malaria and leishmaniasis as well\(^\text{248,245}\); no such reactivity was observed in this study, further strengthening previous findings that an anti-Sm antibody response is not essential for the appearance of anti-hHb antibodies. In patients of both leishmaniasis\(^\text{228}\) and malaria\(^\text{248}\), auto-reactive antibodies of both the IgG and IgM isotype
de~onstrate an increase. Anti-hHb reactivity in leishmania patients, however, was mainly IgG-mediated. While patients harboring P. vivax demonstrated a similar preferential increase in IgG anti-hHb titres, those infected with P. falciparum showed equivalent increases in IgG and IgM anti-hHb reactivity. The reason for these differences, as well as the biological significance of the observation, is at present unknown. The data indicated that anti-hHb autoimmune antibody responses may also occur in non-autoimmune pathologies characterized by extensive hemolysis. It would be of interest to determine whether they are present in diseases in which significant hemolysis is not observed.

NZB/W F1 mice have been extensively employed in the study of lupus and serve as a good model of human disease, since the kinetics of disease onset and subsequent pathologies appear similar to that seen in human lupus. Even though hHb and mHb share a high degree of homology, it was essential to use mHb as a target to ascertain whether or not auto-reactivity to the molecule existed in NZB/NZW F1 animals. Hb from NZB/NZW F1 was purified to homogeneity and characterized by mass spectrometry, HPLC and N-terminal sequencing. Anti-mHb titres exhibited an age-dependent increase, in general consonance with the appearance of anti-self and anti-nuclear reactivity, thus demonstrating a relationship with disease onset (Figure 10a-c). As previously reported in MRL pr/lpr mice, anti-Sm antibody titres also demonstrated an age-related increase, though a slightly extended lag period in their appearance (in comparison with the appearance of anti-mHb antibodies) was observed (Figure 10d). Titres to individual Hb subunits were also ascertained, in an effort to establish whether a particular subunit was preferentially targeted. Though the beta subunit appeared to be more antigenic (Figure 11a, b), the auto-antibody response to the individual subunits was lower than to the whole molecule, possibly indicating the partial dependence of reactivity upon Hb conformation.

In autoimmune diseases, the sequestration of auto-antibodies or T cells in various organs is associated with specific pathologies. For example, anti-dsDNA can be detected in the kidneys and may be linked to the onset of nephritis. In addition, such antibodies have been shown to mediate neuronal death in the CNS, resulting in memory loss. Similarly, transplacental transport of anti-Ro and anti-La antibodies, followed by their binding to fetal cardiac tissue, has been linked to neonatal lupus. The migration of activated CD8+ T cells across the blood brain barrier has
been associated with symptoms associated with multiple sclerosis \(^{250}\). Hb is known to mediate damage to kidneys \(^{214}\) and CNS \(^{215,216,217}\). The involvement of the lungs in SLE is also a relatively common phenomenon \(^{36}\) and this organ is also the venue of Hb-mediated gaseous exchange. Anti-mHb deposition in organs frequently targeted in lupus was therefore studied as a function of age, in both autoimmune and non-autoimmune prone animals (Figure 11c). mHb-reactive antibodies could be eluted (by incubation in a low pH buffer) from the kidneys, lungs and brains of NZB/W F1 animals, and titres appeared to demonstrate age-dependence, with variations in kinetics; while in the lungs, there appeared to be a steady increase of anti-mHb reactivity, titres in the kidneys and the brain registered a significant rise only after ten months of age. Nevertheless, the appearance and presence of anti-mHb in different organs broadly correlated with the onset of clinical symptoms. Similar low pH organ eluates from old BALB/c mice did not demonstrate the presence of antibodies reactive to mHb. The specificity of such reactivity to the autoimmune strain, as well as the kinetics of appearance of reactivity as animals age, point to possible clinical relevance.

To investigate anti-Hb autoimmune antibody responses in further detail, monoclonal antibodies were generated. B cells isolated from SLE patients (in the midst of a disease "flare") were transformed with Epstein Barr Virus as part of another study. Cells secreting antibodies reactive to hHb were “fused” with an appropriate partner and the human monoclonal antibody KV2C8 was generated. Six murine monoclonal anti-mHb antibodies were generated using spleen cells sourced from aging NZB/W F1 mice. Five murine monoclonal antibodies were of the IgM \(x\) isotype, while the sixth was of IgA \(x\) isotype.

As discussed above, extensive hemolysis characteristic of lupus and other diseases leads to the release of free Hb. Serum levels of the Hb-binding protein Hp fall to low levels in diseases characterized by extensive hemolysis, thereby affecting Hb clearance. In an oxidative environment such as that seen in lupus and in other diseased states, it is postulated that the iron in the heme moiety is converted from the ferrous (Fe\(^{2+}\)) to the ferric (Fe\(^{3+}\)) form, resulting in the formation of metHb. Indeed, presence of oxidatively modified Hb in CSF has been demonstrated under conditions of oxidative stress after hemorrhage \(^{251}\). MetHb can elicit a number of biological effects; for example, it causes a dose- and time-dependent increase in the activation of endothelial cells \(^{252}\). Free heme may be released as well, causing a number of toxic effects \(^{192,214}\). To assess whether the
anti-Hb monoclonal antibodies could distinguish between the ferrous and ferric forms of Hb, metHb was generated upon incubation of Hb with H2O2; the fact that H2O2 is known to exist at increased concentrations *in vivo* in SLE210, made these experiments additionally relevant. Both hHb and mHb were employed. ELISA assays revealed that Antibodies 1C1, 3C4, 3A1 and KV bound the ferrous and ferric forms of Hb to almost the same extent. The data indicated these antibodies bound motifs or epitopes not modified or affected due to the oxidation process. In contrast, Antibodies 1B5, 2A1 and 2C1 demonstrated significantly enhanced recognition of ferric Hb. Patterns of reactivity on murine and human Hb were almost the same for all antibodies, validating the results (Figure 15a). This is an observation of significance and implies that a disease-directed change in a self-protein is giving rise to structural neo-epitopes recognized by disease-related monoclonal auto-antibodies.

Cross-reactivity of the monoclonal antibodies with other heme-containing proteins (cytochrome c and myoglobin) and with heme was then assessed (Figure 15b). Myoglobin was specifically chosen as it exhibits high structural similarity with Hb subunits191. Antibody 2C1 appeared to be the most specific to Hb, demonstrating minimal binding to the other moieties; interestingly, it was also one of the antibodies which bound Fe^{3+} Hb better than Fe^{2+} Hb. Antibody 3A1 demonstrated reactivity towards Hb and heme; the other antibodies exhibited varying degrees of cross-reactivity towards the other moieties, as described in detail in the Results section. Therefore the data demonstrated that, even in this limited analysis of cross-reactivity, while one antibody bound Hb with high specificity, others were more broadly reactive.

Free Hb is normally bound by Hp which aids in its clearance and prevents the Hb-mediated toxic events195. It has been shown that binding of Hb to Hp stabilizes the former. Hp shares structural similarities with IgG and so can be considered a “natural antibody” to Hb. In a previous study, antibodies to Hb did not exhibit the stabilizing effect as did Hp, and bound Hb on a site distinct from Hp253. In the present study, experiments were carried out to ascertain whether the binding of Hp to Hb could prevent the binding of the monoclonal antibodies to Hb. Given the cross-reactive nature of some of the antibodies, it was first important to ascertain whether they demonstrated binding towards Hp as well; with the experimental design employed, such reactivity would have vitiated the results, making the data un-interpretable. None of the monoclonal antibodies bound
Hp. Subsequent competition assays revealed that for all antibodies, Hb-Hp association did not affect Hb-Antibody interaction, possibly indicating that the binding sites of Hp and the anti-Hb auto-antibodies on Hb do not overlap. No conclusive results could be obtained for the human monoclonal antibody KV due to unexpectedly high binding of the anti-human Ig reagent with Hp. Similarly, commercial, polyclonal antibodies directed against the alpha and beta subunits of Hb unexpectedly demonstrated significant reactivity towards Hp, precluding interpretation of the data (Figure 16b).

Experiments were then conducted to assess whether the antibodies demonstrated preferential binding towards either the alpha or the beta subunit of Hb (Figure 16c). In spite of high degree of structural similarities between the two subunits, most antibodies could indeed distinguish between the two. Antibodies 1C1, 2A1, 3C4, 3A1 and KV recognized beta subunit better than the alpha subunit. While Antibody 1B5 could not distinguish between the two subunits, Antibody 2C1 was the only antibody which bound the alpha subunit to a greater extent. It was of interest to note that Antibody 2C1 also appeared to be the most specific in the assays discussed above.

Upon intra-molecular epitope mapping using contiguous, non-overlapping peptides representing the alpha and beta subunits of Hb as targets in ELISA, differing results were obtained for the different antibodies (Figure 17, Figure 18). While some antibodies were essentially non-reactive (Antibodies 3A1 and KV), another was predominantly reactive towards one peptide (Antibody 2C1, the relatively Hb-specific monoclonal antibody), while another (Antibody 2A1) preferentially bound two peptides. Even though Antibody 1B5 appeared to exhibit poly-reactive behavior, binding preferences for certain peptides were apparent. When the binding specificities of all antibodies were considered, the peptide 100-119 of alpha subunit and 100-119 of the beta subunit appeared to be recognized by most antibodies. These regions have been found to be immunodominant in previous published reports as well.\textsuperscript{218,219,254} Competition analysis between different reactive peptides, as has been done for polyreactive human monoclonal antibodies reactive to Ro60\textsuperscript{255}, would further validate these findings. Non-reactivity of Antibodies 3A1 and KV towards the peptides may be due to the fact that the conformational epitopes they recognize do not find adequate representation in the peptides employed, or due to the inadvertent destruction of linear epitopes caused by current peptide design. The non-reactivity of commercial, polyclonal
sera against the alpha and beta peptides, however, is surprising. It would be interesting to see if the immunization of animals (both non-autoimmune prone, and those prone to develop autoimmunity) with these peptides (as opposed to the entire Hb) results in the generation of autoimmune responses, as was observed upon immunization of SmD peptides\textsuperscript{112}. If indeed such responses occur, postulates of crypticity and molecular mimicry could be put forth.

As previously indicated, this study was initiated on the observation that an apoptotic cell-specific human monoclonal antibody exhibited frank cross-reactivity to hHb. By applying converse logic, the reactivity of the established anti-Hb monoclonal antibodies towards cellular proteins was assessed. Upon FACS analysis, none of the antibodies, including those that appeared to be polyreactive on previous analysis, bound the surface of healthy, non-permeabilised cells. These results ruled out non-specific "stickiness" being responsible for reactivity. In contrast, all antibodies (with the possible exception of Antibody 2C1) demonstrated significant reactivity towards cells that had been previously permeabilised (Figure 19). This data implied that, in several instances, antibodies that bound Hb also had the capacity to bind cellular antigen(s), leading to the postulate that at least some anti-Hb antibody specificities may arise due to cross-reactivity to such antigen(s), or vice-versa.

Confocal analysis (Figures 20-25) confirmed these results; while none of the antibodies bound non-permeabilised cells, most demonstrated avid intracellular reactivity, binding cytoplasmic and/or peri-nuclear moieties. Antibody 2A1 appeared to also bind antigen(s) sequestered near the cell membrane, while antibody 3A1 appeared to additionally bind nuclear antigen(s). In contrast with these results, commercial polyclonal antibodies against the alpha and beta subunits of Hb, employed here as controls, did not bind to either non-permeabilised or permeabilised cells, indicating differences between some auto-immune and non-autoimmune anti-Hb responses. Interestingly, in consonance with FACS results, Antibody 2C1 also did not appear to recognize cellular antigens even upon permeabilised cells, once again distinguishing it from the other antibodies.
During apoptosis, blebs containing a number of auto-antigens such as Ro, DNA and La are extruded from the cell surface\textsuperscript{256}, and apoptotic cells are thought to constitute an antigenic insult for the development of lupus in genetically susceptible individuals\textsuperscript{131,257}. In furtherance of the hypothesis that cross-reactivity with cellular antigens, as well as the process of apoptosis, may be responsible in part for anti-Hb responses, reactivity of the monoclonal antibodies upon apoptotic cells was assessed using two-colour flow cytometry, employing the antibodies as well as the phosphatidylserine binding protein Annexin-V (Figure 26). In consonance with their reactivity patterns on permeabilised cells, Antibodies 1C1, 1B5, 2A1, 3C4, 3A1 and KV preferentially recognized cells that also bound Annexin-V, which demarcated them as being apoptotic. Although there existed cells that bound Annexin-V but not antibodies, cells binding only the antibodies and not Annexin-V were relatively rare.

Antibody 2C1 demonstrated minimal binding to apoptotic cells, as evident from the lack of a significant number of cells binding both the antibody and Annexin-V; these results once again demonstrated the antibody’s unique properties. To re-iterate: Antibody 2C1, unlike the other antibodies, did not cross-react with either heme, or the heme-containing proteins myoglobin and cytochrome c; it was the only antibody that preferentially bound the alpha subunit of Hb; it was the only antibody that uniquely bound a single peptide from the alpha subunit (peptide 110-119); and it was the only antibody to demonstrate poor recognition of cellular antigens, as well as of antigens extruded upon cells undergoing apoptosis.

As part of another study, a panel of murine monoclonal antibodies specifically targeting apoptotic cells had been established. To further probe the link between apoptosis and anti-Hb reactivity, the ability of these antibodies to bind Hb was assessed; significant binding was observed with several antibodies, with antibodies of the IgM isotype demonstrating higher activity (Figure 27). Previous work with the human and murine apoptotic-cell specific antibodies had established their polyreactive nature; irrespective of isotype or the presence of somatic mutations in the complementarity determining regions (Reference 67 and unpublished observations). The anti-Hb monoclonal antibodies revealed a similar polyreactive nature when reactivity was assessed upon a panel of recombinant auto-antigens, with individual differences apparent (Figure 28). These reactivities, on selected auto-antigens were only indicative in nature; more comprehensive analysis
would be required to delineate the extent and the nature of the non-Hb cross-reactive epitopes within cells. A beginning has been made in this regard; Western blots using three representative antibodies (Antibodies 1C1, 1B5 and 2C1) to probe reactivity upon SP2/O lysates further revealed intermolecular cross-reactivity of Antibodies 1C1 and 1B5; the nature of the reactivity appeared to be distinct however. Antibody 2C1 did not bind any antigens in the lysate, consistent with previous observations. On two-dimensional Western blotting using Antibody 1B5, three major spots were obtained, which await characterization.

Analysis thus far had demonstrated beyond doubt that Hb was antigenic, in both murine and human disease states. Since antigenicity does not necessarily imply immunogenicity, it was important to determine whether Hb was immunogenic in a murine strain genetically pre-disposed to systemic autoimmunity. Both Fe$^{2+}$ mHb and F$^{3+}$ mHb were employed to immunize 2-month old (pre-autoimmune) NZB/W F1 mice, since previous data had shown that some monoclonal antibodies derived from these animals displayed the capability of discriminating between the two forms. In addition, an aggressive adjuvant was employed to maximize the chances to break tolerance. Immunization with neither Fe$^{2+}$ mHb nor F$^{3+}$ mHb generated early autoimmunity to Hb, Sm (assayed because of data demonstrating a possible association between the two reactivities) or other intracellular antigens, indicating Hb might not be an initiating auto-antigen. Indeed, in a non-autoimmune scenario, Hb is known to be a poor immunogen$^{258,259}$ and is capable of generating immune responses only when it is internally cross-linked$^{259}$ or bound to Hp$^{260}$.

Somatic mutations are known to accumulate in antibody variable regions as immune responses mature. Analysis of $V_H$ and $V_L$ sequences has demonstrated that, by and large, IgG antibodies tend to carry more mutations than IgM antibodies, although exceptions do occur. The CDRs tend to contain more replacement mutations (which result in a change in amino acids) than silent mutations (which result in no such change)$^8$.

Several reports exist describing the presence of somatic mutations in auto-antibodies specific for different auto-antigens$^{261}$. For example, mutations to the amino acid arginine in the CDR3 has been shown to be important for anti-dsDNA and anti-Sm reactivity in both murine and human antibodies$^{262,263,235}$. "Reverse" mutations, or mutations to amino acids such as glycine, lead to a
loss of reactivity to DNA, and addition of arginine residues at specific points cause up to a fifty-fold increase in anti-DNA reactivity. In addition to somatic mutations, the presence of arginine in the CDR3 may also result from N-base addition, or because of unusual reading frames arising due to the D region. Residues surrounding the interacting amino acids have been shown to influence reactivity; it was found that when aspartic acid residue was incorporated close to arginine residue, there was stabilization of charges, reducing DNA binding\(^{17}\).

Auto-antibody responses in lupus have been further characterized, and additional defects enumerated. In the MRL/lpr animals, a decrease in the kappa light chain rearrangement and RAG expression is observed\(^{264}\). Antibodies derived from SLE patients demonstrate a decreased CDR3 length\(^{53}\). Several studies have shown a skew in the gene families employed by auto-antibodies, an observation that may be reflective of antigenic specificity. Reports suggest that the majority of the IgM auto-antibodies have VH regions belonging to the J558 family, while IgA antibodies tend to employ 7183 family. Biased use of the J chain has also been observed, in both heavy and light chains; most IgMs use the J\(_{H}4\) gene family and IgAs the J\(_{H}1\) family. The J\(_{K}\) family usage is skewed towards J\(_{K}1\) and J\(_{K}5\) for IgMs and J\(_{K}4\) for IgA auto-antibodies\(^{262,263}\). The human data suggests a bias towards VH3 and VH 4-34 gene family usage\(^{265}\).

The heavy and light chain variable regions of the anti-Hb monoclonal antibodies were sequenced subsequent to PCR amplification using specific primers. Sequences were analyzed for family usage, as well as for the presence of somatic mutations. The analysis has been summarized in the Tables 1a and 1b. Antibodies 1C1 (IgA\(\kappa\)) and 1B5 (IgM\(\kappa\)) both used the 7183 heavy chain Ig gene family, demonstrating the closest homology with the same germline gene (VH7183.a19.31); as indicated above, the family is known to be employed by other auto-antibodies. The other murine anti-Hb antibodies employed the J558 gene family\(^{262}\), albeit demonstrating closest homologies with different germline genes. As J558 corresponds the VH1 family and 7183 to the VH5 family, these two gene families of the heavy chain appeared to be over-represented in anti-Hb responses. While use of the D segment varied widely, three murine antibodies (Antibodies 1C1, 2C1 and 3A1) employed the J\(_{H}1\), two the J\(_{H}2\) (Antibodies 2A1, 3C4) one (Antibody 1B5) the J\(_{H}4\) (1B5) gene segment. Varied use of the light chain variable genes was observed, as was the employment of the J segment; one antibody (Antibody 1C1) used as J\(_{K}1\), two (Antibodies 2A1 and 3A1) J\(_{K}2\),
<table>
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<th>GENES SEGMENTS</th>
<th>REPLACEMENT/ SILENT MUTATIONS IN CDR</th>
<th>REPLACEMENT/ SILENT MUTATIONS IN FWR</th>
<th>MUTATIONS PER UNIT LENGTH IN CDR</th>
<th>MUTATIONS PER UNIT LENGTH IN FWR</th>
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Table 1a: Anti-Hb monoclonal antibody germline gene segments (listed as V, D, J regions for the heavy chains and V, J regions for the light chains) and mutation analysis. ∞ indicates division by zero.
Table 1b: Anti-Hb monoclonal antibody germline gene segments (listed as V, D, J regions for the heavy chains and V, J regions for the light chains) and mutation analysis. ∞ indicates division by zero.
one (Antibody 1B5) Jα4 and two (Antibodies 2C1 and 3C4) Jα5. The length of the CDR3 in the heavy chain varied from antibody to antibody, and demonstrated no apparent correlation with antibody specificity or degree of cross-reactivity, as has previously been reported\(^5\); while in Antibodies 1B5, 2A1 and 3C4, it was shortened to 7, 8 and 7 residues respectively due to exonuclease activity, other antibodies (including Antibody 2C1, which appeared relatively more specific to Hb, as discussed above) demonstrated a longer length. In other antibodies, no significant deviations were observed from the germ line sequence length.

As indicated above, mutations to arginine, or non-encoded junctional additions of the amino acid, have been associated with anti-dsDNA reactivity\(^1\). In the present study, mutation or non-encoded addition to the amino acid glycine in the CDR3 occurred in six heavy chains (Antibodies 1C1, 2A1, 2C1, 3C4, 3A1 and KV) and one light chain (Antibody 1C1). In addition, two sequences (the heavy chain of Antibody 1B5 and the light chain of Antibody 3A1) contained a germline-encoded glycine residue in CDR3. Thus, a total of nine out of thirteen sequences analyzed contained the amino acid glycine in CDR3. While mathematically this may appear a significantly high number, actual proof of the influence of the residue on anti-Hb specificity would have to await mutation analysis.

Two analyses were carried out to study the relative numbers of somatic mutations in the CDR regions and FWR. Firstly, attempts were made to calculate the ratio of the replacement to silent mutations in the CDR and FWR regions. Random mutation processes produce replacement to silent mutation ratios of 2.9 or lower\(^2\). Replacement mutations per unit length (amino acid) were also independently calculated for the CDR and FWR regions, and a ratio of these values determined\(^3\); a value greater than one would indicate that somatic mutations were indeed preferentially directed to the CDR regions.

For heavy chain of Antibody 1C1, replacement (R) to silent (S) mutation ratios for the FWR and CDR regions were 3 and 3 respectively, indicating their non-random nature. Further analysis revealed that the CDR regions carried 2.32 times more replacement mutations per unit length than the FWR regions. For the light chain, the R/S ratio was 3 for the CDR, whereas the FWR was non-mutated. Analysis of the heavy chain of Antibody 1B5 revealed a slight increase in the propensity
of R mutations to be directed to the CDR, with a value of 1.69 being obtained for the ratio of R mutations per unit length in the CDR versus FWR. The light chain of Antibody 3A1 too showed a similar propensity, with the ratio being 1.36. The enumeration of the mutations conducted above is conservative as far as its relevance to anti-Hb specificity is concerned due to two reasons; firstly, in several instances, analysis was hampered by the denominator in the calculations being zero, indicated by the symbol "∞" in Tables 1a and 1b. Secondly, non-encoded amino acids added at the junctions, which in all probability would influence antibody specificity, were not included in the analysis.

Upon release from RBC, free Hb first comes into contact with leukocytes and endothelial cells\textsuperscript{236}. These cells can alter the behavior of other cell types by causing the secretion of IL-6, IL-8 and TNF-α in response to Hb, and the amount of cytokine secreted increases with increasing dose and time of incubation\textsuperscript{237}. Inflammatory cascades set up as a result can have serious implications; clinical trials in patients who received Hb as a blood substitute had to be terminated because of associated fatalities\textsuperscript{237}.

Inflammatory cascades have been implicated in lupus pathogenesis. High levels of serum TNF-α have been reported in patients\textsuperscript{186}, resulting in increased levels of pro-inflammatory cytokines such as IL-1, IL-6 and IL-8\textsuperscript{148}. Anti-IL-6 therapy has beneficial effects in lupus mouse models\textsuperscript{161}. Higher numbers of unstimulated cells secreting IL-6, IL-8 and TNF-α have been observed in SLE patients than in controls\textsuperscript{171}, and high concentrations of these cytokines correlate with increased disease activity. These cytokines have been implicated in the pathology of NPSLE as well; levels of IL-6 and IL-8 were found to be higher in the CSF of SLE patients\textsuperscript{165}.

Whether anti-Hb antibodies could influence Hb-induced secretion of inflammatory cytokines was then investigated. Experiments were carried out at sub-optimal doses of Hb, in order to better elucidate antibody-mediated effects. Antibodies were also individually assayed for their ability to influence cytokine secretion. Interestingly, Antibody 2A1 exhibited the capability to induce the secretion of cytokines. The other anti-Hb antibodies, as well as antibodies of irrelevant specificity employed as isotype controls, did not exhibit such reactivity. Co-incubation of cells with Antibody 2A1 and Hb resulted in significant synergistic effects on cytokine secretion which, once again, was
not observed with any of the other antibodies (Figure 43). The reasons of these effect are currently unclear, but may be related to the antibody’s unique binding characteristics. Antibody 2A1 was the only one to demonstrate dominant reactivity towards peptide 110-119 of the Hb alpha subunit as well as peptide 110-119 of Hb beta subunit, to demonstrate punctate binding to regions close to or at the plasma membrane, and to exhibit a high degree of cross-reactive recognition of the A Protein. Anti-dsDNA antibodies too have been shown to demonstrate such effects; incubation of human mononuclear cells with murine anti-DNA antibodies caused secretion of IL-1β, IL-6, IL-8 and TNF-α; cross-reactive binding to cell surface molecules such as acidic Rib P proteins present on the kidney mesangial cells may initiate such events or these effects might be seen after endocytosis of the antibodies via binding to the membranous brush border myosin. These reports suggest transport of auto-antibodies across the cell membrane into the cytoplasm and the nucleus where they may be involved in altering cellular functions. Although literature also suggests existence of an Fcα/μ receptor (that binds IgM Fc) in the thymus, spleen, liver, kidney, testis, placenta, small and large intestines and on the B cells and macrophages, such a receptor is unlikely to be responsible for the observed effects due to the demonstrated specificity of recognition.

Endothelial cells lining the vasculature are particularly sensitive to Hb-mediated damage as they are in direct and continuous contact with cell-free Hb. Damage is specifically Hb-mediated; other heme-containing proteins like myoglobin and cyt c do not demonstrate such effects. Endothelial cells respond to the presence of Hb by secreting cytokines such as IL-8, a chemo-attractant in a dose and time dependent manner. The cytokines further activate the endothelial cells and cause monocyte adherence to the endothelium, an essential step for migration of cells into tissue. Activation of endothelial cells by TNF-α causes significant attachment of monocytes via adhesion molecules ICAM (Intercellular Adhesion Molecule), VCAM-1 (Vascular-Cell Adhesion Molecule), MCP-1 (Monocyte Chemoattractant Protein-1) and E-selectin. Other reports suggest higher levels of soluble VCAM-1, ICAM-1, E-selectin in the cerebrospinal fluid (CSF) of patients who had subarachnoid hemorrhage, possibly linking free Hb with elevated levels of these adhesion molecules. Increased levels of soluble VCAM-1 have also been found in lupus patients, and levels correlate with disease activity and severity.
Supernatant obtained upon incubation of endothelial cells with Antibody 2A1 did not enhance the transmigration of monocytes as compared to medium and the isotype control antibody. On the other hand, supernatant obtained from endothelial cell cultures incubated with Antibody 2A1 together with Hb significantly enhanced the migration of monocytic cells (Figure 44). Although the precise cytokines and/or soluble adhesion factors responsible for these effects remain to be identified, the data supplement previous results, while shedding additional light on the biological roles Hb-reactive antibodies might play in the patho-physiology of diseases characterized by presence of free Hb.
Summary and Conclusions
SUMMARY AND CONCLUSIONS

A human monoclonal antibody specifically targeting apoptotic cells demonstrated cross-reactive recognition of human hemoglobin (hHb).

Antibodies present in a certain percentage of SLE sera demonstrated high reactivity to hHb in ELISA and upon Western blots. Studies using auto-reactive sera obtained from the CDC (Center for Disease Control and Prevention, Atlanta) from SLE patients demonstrated that only sera containing antibodies to Sm antigens contained antibodies which bound hHb. Subsequent analysis revealed that an anti-Sm antibody response was probably sufficient but not necessary for an anti-hHb antibody response.

Anti-hHb reactivity in human SLE patients could be mediated by both IgG and IgM antibody isotypes. Antibodies in a certain percentage of sera from malaria and leishmaniasis patients were also reactive to hHb (but not to Sm), indicating that aberrant immune recognition of hHb was present in other clinical conditions associated with RBC lysis, enhanced apoptosis and auto-antibody production. The anti-hHb reactivity in these sera was mainly mediated by the IgG isotype; in patients harboring *P. falciparum* however, anti-hHb antibodies of the IgG and IgM isotypes were equally represented.

Mouse Hb (mHb) was purified on a CM-52 ion exchange column; protein purity was assessed by SDS-PAGE and HPLC, and its identity confirmed by N-terminal sequencing and mass spectrometry. Old, autoimmune-prone NZB/W F1 mice demonstrated higher titres of antibodies to mHb than did young animals; the kinetics of appearance of anti-mHb reactivity paralleled the appearance of anti-nuclear antibody reactivity, a hallmark of lupus. Enhanced antibody reactivity to the alpha and beta subunits of hHb was also observed as animals aged. Antibodies to the Sm protein demonstrated a similar age-related increase, albeit with an extended lag-period compared with anti-mHb responses. Low-pH eluates obtained from the lungs, brain and kidneys from NZB/W F1 demonstrated age-dependent increases in antibodies reactive to mHb, while eluates from tissues derived from old BALB/c animals did not contain...