Summary and Conclusions
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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...
antibodies which bound mHb, potentially implicating anti-Hb auto-antibody responses in end-organ pathology.

A certain percentage of supernatants of 456 EBV transformed B cell lines derived from SLE patients demonstrated the presence of antibodies reactive to hHb. A human monoclonal antibody KV (IgM\text{a}) reactive towards hHb was established upon fusing cells with the heteromyeloma K\text{c}H\text{s}/B\text{s}. Spleen cells of aging NZB/W F1 mice were fused with the B-cell myeloma SP2/O. Six monoclonal antibodies (1B5, 2A1, 2C1, 3C4, 3D1 [all IgM\text{a}], and 1C1 [IgA\text{a}]) were established. While some antibodies were equally reactive towards ferrous (Fe^{2+}) Hb and ferric (Fe^{3+}) Hb, others had the capability of distinguishing between these two forms. Antibody 2C1 appeared to be relatively Hb specific, whereas other antibodies demonstrated varying degrees of cross-reactivity towards heme and other heme-containing proteins cytochrome c and myoglobin.

Competition studies revealed that Hp and the monoclonal antibodies bound distinct sites on Hb. Most antibodies demonstrated preferential reactivity towards either Hb-alpha or Hb-beta, while Antibody 1B5 bound them both equally. Studies using synthetic peptides spanning alpha and beta Hb helped elucidate epitopes recognized by some anti-Hb monoclonal antibodies; Antibody 2C1 predominantly bound to a single peptide, while Antibody 2A1 bound two non-homologous peptides, one each on the alpha and beta subunits of Hb, demonstrating intra-molecular cross reactivity. Other antibodies were either poorly reactive, or demonstrated a more poly-reactive binding pattern.

Though antibodies were non-reactive towards cell surface moieties, they (with the exception of Antibody 2C1) demonstrated cross-reactive recognition of predominantly cytoplasmic antigen(s) on FACS and confocal microscopy. Reactive antibodies also bound epitopes exposed on the surface of cells undergoing apoptosis. The cross-reactivity of the anti-Hb monoclonal antibodies with non-Hb self antigens was further elucidated by ELISA on selected recombinant auto-antigens; Ro60, La, SmD, SmB and the U1RNP A protein were differentially recognized. The IgA Antibody 1C1 reacted to different moieties than did the
IgMs upon Western bolt analysis on cellular lysates. Further elucidation of cross-reactive moieties would help elucidate the etiology of anti-Hb antibody responses.

Immunization of pre-diseased, autoimmune prone mice with either ferrous or ferric mHb did not result in the enhanced generation of anti-mHb or other anti-self antibody responses, indicting that mHb was probably not the primary immunogen in the generation of anti-mHb responses.

The light and heavy chain variable region genes of the anti-Hb monoclonal antibodies were sequenced. Comparisons with closest germline sequences revealed that, while some antibodies appeared to be essentially germline encoded, others carried somatic mutations. Nine of thirteen CDR3s were found to contain a Glycine residue, either germline encoded, or arising as a result of somatic mutation or a non-encoded addition at the junctional regions.

Antibody 2A1 stimulated the release of TNF-α, IL-6 and IL-8 from THP-1 (human monocyte) cells; significant synergy in cytokine production was observed when cells were co-incubated with the antibody and Hb. Such synergistic effects were also observed upon incubation of endothelial cells with the combination of antibody and Hb; supernatants arising from such cultures were significantly more efficient in inducing the transmigration of monocytes than antibody or Hb alone. The results reveal that anti-Hb responses may be involved in the amplification of inflammatory cascades in diseases of immune dysfunction.