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Genetic contributions to the autoantibody profile in a rabbit model of systemic lupus erythematosus (SLE)

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Abstract

For the development of rabbit models of Systemic Lupus Erythematosus (SLE), immunoglobulin allotype-defined pedigreed rabbits from the National Institute of Allergy and Infectious Diseases rabbit resource more closely approximate human populations due to their non-inbred pedigreed structure. In an initial study from this laboratory, peptides (SM and GR) from the spliceosomal Smith (Sm) and the NMDA glutamate receptor NR2b, on branched polylysine backbones (BB) elicited antinuclear and anti-dsDNA autoantibodies typical of SLE, as well as seizures and nystagmus sometimes observed as neurological manifestations in SLE patients. This suggested the feasibility of further selective breeding to develop a more reproducible rabbit model for investigations of SLE. Here we report the results of GR-MAP-8 and control BB immunization on autoantibody responses in a group of 24 rabbits specifically bred and developed from parents and ancestors tested for autoantibody responses. The changes in hematological profile and blood chemistry in the experimental rabbits were evaluated along with autoantibody responses. Elevations of total white blood cell (WBC), monocyte, eosinophil and basophil counts that developed following immunizations were moderately influenced by litter and presence of the antibody heavy chain allotype VH1a1. Autoantibody development followed a sequential pattern with anti-nuclear antibodies (ANA) followed by anti-dsDNA and subsequently anti-Sm and anti-RNP similar to SLE patients. High autoantibody levels to one autoantigen were not always associated with antibody response to another. Female rabbits had higher prevalence and levels of autoantibodies similar to human SLE. Higher autoantibody levels of anti-dsDNA and -ANA were observed among some full sibs and the presence of high responder ancestors in the pedigree was associated the augmented responses. We observed significant association between highest antibody responses to GR-MAP-8 and highest anti-dsDNA levels. Naturally occurring autoantibodies were found in some pre-immune sera and some unique ANA fluorescent staining patterns within the experimental group were observed. Background immunofluorescence in pre-immune sera, distinct patterns of programmed autoantibody responses unique among individual rabbits may have been modulated by genetic constitution, gender and environmental factors including exposure to antigens. The high incidence and intensity of autoantibody responses among descendants of high responders suggest that there may be an additive mode of inheritance with high heritability. It is conceivable that further rigorous pedigree selection for autoantibody responses could lead to development of rabbit models with spontaneous occurrence of SLE like serology and disease phenotypes.

Keywords

Rabbits; Autoantibodies; Antibody heavy chain allotypes; Genetics; Lupus

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1. Introduction

Systemic lupus erythematosus (SLE) has a multifactorial etiology with both genetic predisposition and environmental influences. The disease is characterized by systemic autoimmune responses that may include rash, inflammatory arthritis, generalized glomerular nephritis and renal failure. Neurological manifestations range from cognitive impairment to severe and sudden psychosis, seizures, and strokes. Autoantibodies specific to SLE appear several years before the onset of clinical disease and at least in one example, SLE-specific autoantibody was detected 9.4 years before the clinical onset (Arbuckle et al., 2003). Autoantibodies contribute to the molecular pathogenesis, tissue injury, organ damage, clinical signs, disease progression and mortality in SLE. Anti-dsDNA antibodies are believed to play a role in molecular pathogenesis of lupus nephritis (see recent commentary by van Bavel et al., 2007) and some neuropsychiatric (NP) forms of SLE (Sakic et al., 2005, Schenatto et al., 2006). Disease progression is associated with autoimmune responses to increased numbers of autoantigens and to more epitopes within each autoantigen (epitope spreading). The presence of autoantibodies in healthy individuals complicates serological diagnosis of autoimmune diseases (Quintana et al., 2003). The authors could distinguish cyclophosphamide-accelerated diabetes susceptible and resistant mice based on their autoantibody repertoire prior to induction of diabetes. This has great potential to predict future disease outcomes based on the preexisting antibody repertoire (Quintana et al., 2004).

The genetics of autoimmune diseases has been extensively reviewed (Nath et al., 2004; Gregersen and Behrens, 2006). Contributions of both genetic and environmental factors to SLE development suggest an additive model of inheritance based on genetic load in mouse strains (Mohan, 2006). Polygenic inheritance of SLE susceptibility and phenotype, and interaction of genes at several polymorphic loci, contributing additively to the inheritance has also been postulated in man (Nath et al., 2004; Wang et al., 2007). Polygenic inheritance for human SLE with a heritability of 43.6% among Chinese populations was recently suggested (Wang et al., 2007). Ten to 20 % of all SLE patients have a first or second degree relative with SLE (Hochberg, 1997). Epidemiological analyses and genome wide studies in human populations all suggest a strong genetic component for SLE susceptibility with involvement of multiple genes (Ferreiros-Vidal et al., 2007; Graham et al., 2007; Gregersen and Behrens, 2006; Lindqvist and Alarcon-Riquelme, 1999).

The presence of high levels of serum antinuclear antibodies (ANA) has also been detected before the onset of disease in mouse models of SLE (Xie et al., 2002). Murine mutant, transgenic and knockout models of SLE have contributed substantially to our understanding of molecular pathogenesis and underlying genetic defects leading to SLE (Stoll and Gavalchin, 2000). However, an inherent weakness of the murine models lies in their inbred population structure quite unlike human populations, necessitating development of animal models with a population genetic structure more similar to human populations. This has become more relevant as genomic and proteomic approaches to elucidate the molecular pathogenesis and disease progression of SLE are becoming available.

For the development of rabbit models of SLE, the National Institutes of Allergy and Infectious Diseases (NIAID) rabbit resource offered animals that more closely approximate human population structure due to their non-inbred pedigreed nature. These immunoglobulin allotype-defined pedigreed rabbits have the unique potential to be selected and bred as models for SLE. An initial study aimed at development of models of SLE in pedigreed rabbits, reported autoantibody responses similar to human SLE, when rabbits were immunized with peptides synthesized on branched polylysine backbones (BB), with four (MAP-4) or eight (MAP-8) peptides. Immunizations with MAP bearing a peptide from the spliceosomal Smith antigen (Sm B/B') [SM-with sequence similarity to a peptide sequence from the Epstein-Barr virus

(EBV)-encoded nuclear antigen (EBNA 1)] confirmed and extended an earlier report that used the SM peptide (James et al., 1995). MAP with either the SM peptide or a peptide found in the NMDA glutamate receptor NR2b (GR), elicited antinuclear, and anti-dsDNA antibodies in a total of 17 out of 24 immunized rabbits (Rai et al., 2006). This suggested the feasibility of further selective breeding of the rabbits of this colony to develop a more reproducible rabbit model for investigations of SLE.

For further investigations we chose to immunize with the GR-MAP-8 peptide that induced strong autoantibody responses in the earlier studies (Rai et al., 2006). The GR peptide sequence (DEWDYGLP) is related but not identical to the peptide DWEYS used by others based on the observations of Diamond and coworkers (Gaynor et al., 1997; DeGiorgio et al., 2001; Sharma et al., 2003), who suggested that a peptide mimotope which was bound by anti-dsDNA mAb R4A as well as other anti-dsDNA antibodies was related to a sequence in the NMDA (NR2b) receptor (reviewed in Rice et al., 2005). It has been suggested that such cross-reacting antibodies contribute to neurological or psychiatric manifestations in some lupus patients (NP-SLE) (Omdal et al., 2005; Kowal et al., 2006; Hanly et al., 2006).

Here we report the results of GR-MAP-8 immunization on autoantibody responses in a group of rabbits specifically bred and developed from parents and ancestors tested for autoantibody responses. GR immunization was found to elicit anti-dsDNA and -ANA autoantibody responses in the experimental group. Results of this study also indicate potential for the development of rabbit lines that may serve as models of spontaneous SLE.

2. Materials and methods

2.1. Animals

Rabbit experimentation and immunizations described here were reviewed and approved by the animal care and use committees of NIAID/National Institutes of Health and of Spring Valley Laboratories where the rabbits were housed. A total of 24 rabbits of both sexes between 6 months and one year of age were used. The designations, sexes and allotypes of experimental rabbits belonging to eight litters are summarized in Table 1. These rabbits were relatives and descendants bred and developed from the previous lupus experimental groups, based on autoantibody responses.

2.2. Pedigree analyses

Breeding and experimental records on the breeding colony were maintained and data entered into a custom designed 4th Dimension relational database (Tiller Systems, Brunswick, MD). Information from the database was exported into the Pedigree-Draw program (Jurek Software, Cottage Grove WI).

2.3. Antigens

The GR peptide sequence DEWDYGLP corresponding to a known rabbit sequence within the extracellular region of the 2b subunit of neuronal post-synaptic NMDA receptor was synthesized on branched lysine MAP-8 backbone (Anaspec Inc, San Jose, CA). MAP-8 without peptide served as control antigen and all synthesized structures were analyzed by HPLC and MALDI-TOF mass spectroscopy (National Institutes of Health peptide synthesis and analysis unit, Research Technologies Branch, Rockville, MD).

2.4. Immunizations

The 24 rabbits belonging to both sexes (Table 1) were arbitrarily divided into two groups of 12 in order to immunize and collect samples on successive days. Eight animals of each group were immunized with GR-MAP-8 and 4 with BB antigens. Each of the rabbits received primary

s.c. immunization with GR-MAP-8 peptide or control BB at 0.5 mg/0.5 ml in borate buffered saline (pH 8.0), emulsified with an equal volume of complete Freund's adjuvant (CFA) (product F 5881, Sigma, St. Louis MO). Rabbits were boosted at 3 wk intervals with the same antigen concentration emulsified in incomplete Freund's adjuvant (IFA) (product F 5506, Sigma). Rabbits received a total of 7 boosts. Sera were collected prior to immunization (pre-immune) and 7 or 8 days after each boost, aliquoted and stored frozen prior to assays.

2.5. Clinical evaluations

Hematology using a Bayer Advia. model 120 hematology analyzer and blood chemistry assessments of each rabbit were carried out in an approved Veterinary diagnostic laboratory (Antech Diagnostics, Lake Success NY). At the initiation of the study, rabbits were selected that had hematology and blood chemistry values within the reference range. They were then regularly monitored for clinical signs of disease and were subjected to periodic health checks. Whole blood and sera were collected 7 or 8 days after the third, fifth and seventh boosts from each animal for hematology and blood chemistry assessments (Antech Diagnostics).

2.6. Peptide ELISA

Serum antibody responses to immunizations with GR-MAP-8 and control BB antigens were assayed by standard solid-phase ELISA. Polystyrene 96-well plates (Corning; Catalog No. 3590) were coated with 50 μ l per well of 10 μ g/ml GR-MAP-8 in bicarbonate buffer (pH 9.6) and incubated at 4°C overnight. Plates were washed three times with PBS (pH 7.2) containing 0.1% Tween 20 and blocked with 100 μ l of blocking solution for 1 h at 37 °C (Quality Biological Inc., Gaithersburg, MD). Wells were incubated for 1 h at 37°C with sera titrated by four fold dilutions in blocking solution, washed five times, incubated for 1 h at 37°C with 50 μ l of a 1/2000 dilution (0.4 ng/ μ l), of affinity purified HRP-conjugated goat anti-rabbit IgG (H + L) secondary antibody (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA) developed with 3,3', 5,5'-tetramethylbenzidine (TMB) (Inova Diagnostics, San Diego, CA) and the resulting OD read at 450 nm.

2.7. Autoantibody assays

2.7.1. Anti-dsDNA ELISA—Autoantibodies to dsDNA were assayed using the QuantaLite dsDNA assay (Inova Diagnostics), with calf thymus DNA antigen designed for human serology. The manufacturer's instructions were followed except that anti-rabbit secondary antibody was substituted. Briefly, 100 μ l 1/101 diluted rabbit sera were added to antigen coated wells and incubated for 30 min at room temperature (RT). Wells were washed, incubated for 30 min at RT with 1/2000 diluted secondary Ab, HRP-goat anti-rabbit IgG Fc (Jackson ImmunoResearch Laboratories) and then developed with TMB for reading OD at 450 nm. In this and other autoantibody ELISA, data were expressed as the OD of post-immune minus the OD of pre-immune serum from each individual animal (Δ OD).

2.7.2. ANA, RNP and Sm ELISA—Antinuclear antibody ELISA were carried out using a commercially available ANA kit (QuantaLite ANA). Briefly, 100 μ l of 1/41 diluted rabbit sera in sample diluent were incubated in the ELISA wells at RT for 30 min. followed by washing three times and incubation with 1/8000 diluted secondary Ab, HRP-goat anti-rabbit IgG Fc (Jackson ImmunoResearch Laboratories) and then developed with TMB for reading OD at 450 nm. Data were expressed as Δ OD. Assays of sera of the experimental rabbits for autoantibodies to RNP and Sm characteristically used in human SLE diagnosis were similarly conducted using commercially available kits (QuantaLite RNP and Sm kits, Inova diagnostics). The manufacturer's instructions were followed with appropriate modifications of secondary antibodies as described previously (Rai et al., 2006).

2.7.3. Indirect immunofluorescence for detection of antinuclear antibodies

(ANA)—Commercially available slides coated with fixed Hep-2 cells (Antibodies Inc., Davis, CA) were incubated with rabbit antisera diluted 1/10 with 5 % goat serum (Jackson ImmunoResearch Laboratories, Inc.) for 30 min at RT. ANA antibody was detected following 30 min incubation at RT with 12.5 ng/μl FITC goat anti-rabbit IgG Fc (Southern Biotech, Birmingham, AL). Some strongly positive sera were reexamined at 1/40 dilution. Fluorescent binding patterns were photographed under a fluorescence microscope. Patterns were compared with reference pictures provided by Antibodies Inc. Fluorescence patterns of GR- and BB-immunized rabbits along with between animal variability and naturally occurring fluorescence patterns were recorded.

2.8. Statistical Analyses

Student's t test, Pearson's correlation analysis and ANOVA procedures were all performed using SAS v8.0 software. Student's t tests were conducted to examine whether gender or BB versus GR immunization affected hematologic and antibody responses. Pearson correlation coefficients were calculated to quantify how well hematologic and antibody responses were correlated. To examine the effects of family and immunoglobulin heavy chain haplotype on hematologic and antibody responses, a one-way ANOVA was performed.

3. Results

3.1. Clinical findings

All the immunized rabbits showed blood pictures suggestive of chronic inflammatory responses. Table 2 shows mean and highest post-immunization white blood cell (WBC), monocyte, eosinophil and basophil counts and Fig. 1 summarizes the WBC data arranged according to the heavy chain immunoglobulin VH1a allotype of the individual. Hematology and blood chemistry results on samples from rabbits prior to immunization were well within the reference physiological limits (reference range/μl WBC, 4,000–10,000; Monocytes; 0–300, Eosinophils, 0–100; and Basophils, 0–500). Neutrophil and lymphocyte counts remained normal (reference range/μl Neutrophils 1,200–7,000 and Lymphocytes 1,200–7,000). Total WBC counts increased in all animals whether immunized with GR-MAP-8 or with the BB (branched backbone) in CFA and boosted with the same antigen in IFA. Clinical symptoms suggestive of neurological manifestations of SLE such as seizures and nystagmus observed in an earlier study from this laboratory (Rai et al., 2006), were not observed in the present group of experimental rabbits. VHα allotypes (also a possible reflection of linked traits of the haplotype of the rabbit Ig H chains) appeared to influence the leukocyte responses to GR and BB immunizations. Rabbits with a1/a1 allotype had significantly higher total WBC (Fig. 1 and Table 2), compared to rabbits of a1/a2 and a2/a2 allotypes. Sex of rabbit did not significantly influence the leukocyte responses to immunizations. Significance was evaluated by one-way ANOVA.

3.2. Antibody response to GR peptide

The pre-immune sera had no detectable antibodies to GR-MAP-8 peptides and there was no significant anti-peptide antibody in control BB-immunized rabbits with the exception of one BB immunized rabbit (2UA344-1; BB65) that had a slight elevation in OD after the 5th and 7th boosts (0.73 and 0.58, respectively). Antibodies to GR-MAP-8 rose rapidly after immunization. The correlation between GR-MAP-8 antibody responses of individual rabbits after the 3rd, 5th and 7th boost responses was 0.99 ($P \leq 0.0001$). Sex, litter or allotypes were not found to significantly influence antibody response to GR-MAP-8 (Table 3).

3.3. Autoantibody development and correlations

High antibody levels measured by ELISA against one autoantigen were not always associated with similarly high autoantibody responses to another antigen and different litters showed variability in autoantibody levels to dsDNA, Sm and RNP, and ANA in response to immunizations (Table 3).

3.3.1. ANA ELISA—This assay detected some naturally occurring ANA in most pre-immune sera, and one third had OD values greater than 0.5. OD values in pre-immune sera ranged from 0.022 in GR49 to 1.58 in GR61 (Fig. 2). There was a considerable increase in ANA measured after immunizations in all experimental animals (Table 3). The measured serum ANA exhibited an increasing trend through the rabbits' 7th boost. Correlation between ANA autoantibody levels at 3rd, and 5th boosts (0.677) was highly significant ($P \leq 0.01$) and correlation between 3rd and 7th boost (0.434) was significant ($P \leq 0.05$). The shaded cells in Table 3 highlight responses where the Δ OD value was at least one sd above the means of all responses of GR-MAP-8 immunized rabbits in that column.

3.3.2. ANA immunofluorescence assay—In view of the fact that most rabbits had detectable pre-immune ANA by ELISA, we analyzed the same sera by indirect immunofluorescence. Pre-immune sera of 15/24 (62.5%) were negative in the indirect ANA immunofluorescence assay (ANA-IFA) (Table 3). Although ANA-IFA staining was seen with sera from both GR- and BB-immunized animals, the staining patterns observed with the GR- and BB- immunized rabbits differed. Sera from GR animals generally had peripheral or diffuse fluorescence with fine granular or speckling pattern while BB had a peripheral pattern (Fig. 3 and Table 3). In Fig. 3, ANA-IFA patterns observed with sera of typical BB and GR-MAP8 immunized rabbits (panels A1 to A4 and B1 to B4) and atypical patterns (panels C1 and C2 GR59 and panels D1 and D2 BB69) are documented. GR59 serum had atypical cytoplasmic fine speckling pattern not observed in any other rabbits (Fig. 3 panels C1 and C2). BB69 had a discrete speckling pattern not found in other BB or GR animals in both pre-immune and post-immune sera (Fig. 3 panels D1 and D2). Re-examination of the pre-immune and post boost sera of BB69 at 1:40 dilution, (panels E1 to E4) revealed that the pattern seen in pre-immune serum remained but became elevated in titer by the third boost. Generally, littermates had a similar fluorescence pattern. 1UA344 littermates showed less ANA-IFA reaction in both pre- and post-boost sera with the exception of GR57.

3.3.3. Anti-dsDNA autoantibody responses—Considerable between-animal variability existed for anti-dsDNA autoantibodies in pre-immune sera, with OD values ranging from none to 0.98 (Fig. 4 and Table 3). Although many pre-immune sera had autoantibodies to dsDNA, in 12/16 GR- immunized rabbits the autoantibody level to dsDNA increased substantially (Fig. 4). In all the BB animals there was only a marginal increase in measured anti-dsDNA antibody (Table 3). Correlations between anti-dsDNA autoantibody responses at 3rd, 5th and 7th boost were highly significant ($P \leq 0.001$).

3.3.4. Anti-RNP antibodies—Anti-RNP antibody levels in pre-immune sera varied considerably ranging from none to OD 1.08. Anti-RNP responses were elicited in both BB and GR animals. After the 3rd boost, 7 animals' sera gave Δ OD above 1.0, and at the 5th boost, 6 animals had anti-RNP Δ OD above 1.0. This was reduced to 4 rabbits after the 7th boost (Fig. 5A). The highest anti-RNP responses were in UA345 littermates both in GR and BB immunized rabbits and lowest in 1UA344 littermates (Table 3).

3.3.5. Sm ELISA—Sm antibody levels reached their highest by the 3rd boost when 6/12 rabbits had Δ OD above 1.0 but only 3/12 had Δ OD above 1.0 after the 7th boost. Both GR

and BB animals developed high Sm responses. The highest anti-Sm antibody value was seen in GR51. Rabbits of 1YY119 litter had the highest anti-Sm response (Table 3 and Fig. 5B).

3.4. Autoantibody development, sex effects and correlations

Development of autoantibodies appeared to have a sequential chronology with anti-nuclear antibodies (ANA) developing first with 16/24 sera having a Δ OD above 1.0 from the third boost onwards. Four of these responders were BB-immunized (Fig. 2). This was followed by anti-dsDNA antibodies with 5/24 rabbits' sera having Δ OD above 1.0 after the third boost. This increased to 12/24 animals after 5th and 7th boosts (Fig. 4). After the 7th boost, 4/24 rabbits had Δ OD above 1.0 for anti-RNP and anti-Sm antibodies (Figs. 5A and 5B).

Table 4 presents all significant correlations between leukocyte, immune, and autoantibody responses at 1% and 5% levels. The most highly significant leukocyte response correlations were WBC counts at 3rd and 5th, and 5th and 7th boosts and monocyte counts at 3rd and 5th boosts that were correlated with WBC counts at 3rd boost. Correlation between monocyte and basophil counts at 7th boost was also highly significant. Anti-dsDNA levels in pre-immune sera were negatively correlated with GR-MAP-8 antibody development at 3rd, 5th and 7th boosts ($P \leq 0.05$), but correlations between GR-MAP-8 antibody and anti-dsDNA levels during the 5th and 7th boosts were positive and highly significant. Fifth boost anti-dsDNA and ANA levels had a significant ($P \leq 0.05$) correlation ($r = 0.446$), while this correlation was 0.532 ($P \leq 0.01$) at 7th boost. Correlations between anti-Sm and anti-RNP antibody levels at 5th boost and 7th boost were highly significant.

Gender appeared to exert a substantial effect on the highest autoantibody responses. Among 9 rabbits with highest anti-dsDNA levels one standard deviation above mean Δ OD, 6 were females and 3 were males. Similarly, among 13 rabbits with highest ANA IFA responses, 9 were females and 4 were males. Among 8 rabbits with highest RNP and Sm antibody levels 6 were females and 2 were males. Among 55 observations of high autoantibody responses 70.9% were from female rabbits. This is in general, indicative of high autoantibody responses among female rabbits.

3.5. Effect of genetic background on autoantibody responses

Pedigree analyses of experimental rabbits in the context of previous experimental groups and their autoantibody responses revealed a pattern suggestive of genetic influences on autoantibody responses (Fig. 6). In contrast to earlier generations tested in four previous experimental groups the present group was more consistently autoantibody positive although 2UA344-1 and the closely related 1UA344 litter exhibited lower levels of autoantibodies. The 1UA344 litter was sired by 1LL64-3 which had an inbreeding coefficient of 18.8% with the dam 2YY336-4, as the maternal grand sire is again 1LL64-3. Genes from this sire are concentrated in the litter to more than 62.5% and there is a predicted increase in homozygosity of 18.5% of the sire's genes in the litter. This sire has no history of autoantibody responses and further SM-2 and SM-4 non-responder rabbits of the previous SLE experiment contributed to the pedigree of this litter. The highest anti-dsDNA responses were observed in the 2YY299 litter sired by SM-13 (LL191-1) and with paternal and maternal grand sires SM-1 (XX129-3) and SM 15 (2LL179-1). All these sires were high autoantibody responders and SM-1 exhibited seizures (Rai et al., 2006). The highest ANA responses were found in 1YY327 littermates. This litter had a control only injected with PBS (PB45) (1QQ173-1) as sire, that developed spontaneous autoantibodies to SS-A (Ro) and SS-B (La). The paternal sire was again SM13 (LL191-1) and grand sire SM-1 (XX129-3). In addition the maternal dam GR30 (LL108-4) was a high responder.

4. Discussion

We have described here a novel approach to the development of animal models of human SLE by evaluating the autoantibody responses of a group of rabbits specifically bred and developed from parents and ancestors tested for autoantibody responses. This approach allowed us to trace their genetics and immune responsiveness through multiple generations. The proposed polygenic mode of inheritance of SLE resulting from the cumulative interaction of multiple genes at different loci with various loci contributing to pathogenesis and clinical course of disease among human populations (Lindqvist and Alarcon-Riquelme, 1999; Nath et al., 2004; Gregersen and Behrens, 2006; Wang et al., 2007) suggests the value of evaluating autoantibody responses in animal models approximating the genetic structure of human populations. The pedigreed allotype-defined non-inbred rabbits maintained at NIAID provided an alternative to conventional mouse models of SLE (Rai et al., 2006). GR peptide on MAP-8 backbone elicited strong antibody and autoantibody responses confirming the previous report from this laboratory (Rai et al., 2006).

4.1. The Role of Adjuvants

This as well as our earlier study (Rai et al., 2006) and those that previously reported responses to SM-MAP-8 (James et al., 1995), utilized CFA followed by IFA for MAP immunizations. CFA and IFA can induce altered proliferative leukocyte responses and differentiation. Billiau and Matthys, 2001, reported that the CFA-mediated activation of innate immune compartments was important in regulating early induction of autoimmunity and in later phases also provided effector and regulatory cells. Immunization with GR-MAP-8 and the MAP-8 branched polylysine BB resulted in leukocytosis, monocytosis, eosinophilia and basophilia typical of chronic inflammatory responses. The CFA and IFA used in GR and BB immunizations may have generated the elevated leukocyte responses and some autoantibodies in both GR- and BB-immunized rabbits. Genetic backgrounds and the pedigree of the rabbits appeared to modulate the leukocyte responses with rabbits of 1UA344 litter having lower and the 2YY and 1YY119 litters higher leukocyte responses. In mice, Kikuchi et al. (2006) found clear association between percentages of monocytes and serum levels of anti-nuclear antibodies; monocyte levels at 10 months of age and lethal nephritis by 18 months of age were correlated. Rogers et al. (2007) observed that monocytosis was closely associated with autoantibody production in NZB but not BXS mouse models. In our rabbit model, homozygosity for the Ig heavy chain haplotype with the VH1a1 allotype allele correlated with enhanced WBC and monocyte responses suggesting genetic influences of these traits.

4.2. Immunoglobulin heavy chain haplotypes

Polymorphisms at immunoglobulin allotype loci have been speculated to confer selective advantage in a highly prolific species like rabbits resulting in maintenance of allotypic variants over millions of years. Su and Nei (1999) hypothesized that the polymorphisms of the VH1a-allotypes in rabbits have specific adaptive advantages and the three alleles have adapted to cope with three sets of pathogens becoming more or less irreplaceable and hence maintaining the polymorphism over millions of years. Preliminary data from this study limited to a small population, are suggestive of a heavy chain haplotype-correlated leukocyte response to peptide immunization in CFA and IFA among laboratory rabbits. Rather than being strictly explained by the contribution of the VH α allotypic genes, the entire haplotype of linked variable and constant region genes must be considered. A role for rabbit Ig gamma chain polymorphism in maintaining natural selection pressure on wild and domestic rabbit populations has been attributed to the modulation of resistance to bacterial protease degradation of antibody molecules (Esteves et al., 2006). Maintenance of polymorphisms of the IgG hinge region d allotypes in wild populations of rabbits support this view. In the mouse MRL/lpr Fas-deficient model of SLE, Cohen and Eisenberg (1991) originally observed that anti-Sm, anti-chromatin,

and IgM rheumatoid factor (RF) production segregated with immunoglobulin heavy chain b allotype in crosses of heterozygous MRL/lpr (*Igh^j*) x B6/lpr(*Igh^b*) back to MRL/lpr (*Igh^j*) mice. They later showed that in heterozygotes, the anti-histone component of anti-chromatin, and anti-Sm were preferentially skewed toward IgG2a of b allotype. Although they originally hypothesized that certain immunoglobulin variable region genes may be more amenable to autoantibody production, they later studied an *Igh* recombinant locus and suggested the b allotype skewing mapped 3' of most VH genes (Halpern et al., 1992). More recently, Rifkin et al. (2000) showed that IgG2a complexed with typical autoantigens activated RF⁺ autoreactive B cells specific for the allotype of the RF. Activation of dendritic cells with IgG complexes containing nucleic acids was also more effective than complexes with foreign proteins.

4.3. Possible microbial effects on ANA, anti-dsDNA and anti-RNP autoantibodies in pre-immune rabbit sera and correlated WBC and monocyte responses to immunizations

Significant correlation between post-boost leukocyte responses and pre-immune autoantibody levels suggests that further evaluation of the relationships between naturally occurring autoantibodies and post-immunization leukocyte responses in rabbits are needed. The potential protective and anti-microbial specificities of many anti-dsDNA antibodies has been known for many years (Limpanasithikul et al., 1995). The possibility of subclinical microbial infections affecting pre-immune autoantibody levels and their protective role against infections offer a probable explanation for the above phenomena in rabbits. SLE susceptibility and predisposition to high autoantibody responses is often associated with increased resistance to infections contributing to fitness and thereby favored in natural selection. The *sle3* susceptibility locus has been reported to confer enhanced bacterial resistance in mice (Mehrad et al., 2006).

Molecular pathogenesis of SLE initiated by chromatin-immune complexes can involve dual engagement of Fc receptors such as FcγRIII and Toll-like receptor (TLR) 9 as well as another TLR9 independent pathway (Boule et al., 2004). Microbial pathogen associated molecular patterns (PAMPS) that engage TLR or upregulate TLR expression may explain disease flares during infections in patients due to synergistic effects of PAMPS and antigen-autoantibody immune complexes (Leadbetter et al., 2002). TLR7 is a RNA-sensing receptor that may trigger plasmacytoid dendritic cells to produce proinflammatory cytokines including interferon α. Responses to Sm and RNP in this and our previous study, were not correlated with anti-dsDNA responses. In addition, compared to MAP-8 peptide immunization, MAP-4 peptide resulted in fewer anti-dsDNA positive and greater numbers of anti-Sm/Rnp/SS-A/SS-B positive responders (Rai et al., 2006). A differential role of IL6 in regulating these responses in a pristane- induced model of SLE has been observed (Richards et al., 1998), and RNA associated antigens have been shown to activate B cells via combined TLR7-BCR stimulation (Lau et al., 2005). TLR7 duplication in mice with the *yaa* genetic modifier may lead to elevated TLR7 expression and skew autoantibody responses toward RNA-associated antigens (Kumar et al., 2006; Pisitkun et al., 2006). Both these mice with a deficiency of marginal zone B cells and mice with defective MZ B-cell scavenger receptors may be predisposed to spontaneous autoimmune disease (Wermeling et al., 2007).

4.4. Sequential development of autoantibodies

We confirmed previous observations of Rai et al. (2006) that naturally occurring antibodies to GR-MAP-8 peptides were absent from our pre-immune sera. Antibody responses following immunization with GR-MAP-8 peptides peaked by the third boost and highly significant positive correlations between different boosts indicated that peak and persistence was associated. Autoantibody emergence followed a sequential pattern with high prevalence and intensity of ANA responses by the 3rd boost (16/24 animals with Δ OD above 1.0). Anti-dsDNA antibodies began to emerge by third boost (5/24 rabbits with Δ OD above 1.0), but their intensity and prevalence peaked by 7th boost (12 out of 24 rabbits Δ OD above 1.0). Prevalence of anti-

Sm and anti-RNP antibodies was moderate, and limited to 4 out of 24 rabbits at the 7th boost with a peak Δ OD of above 1. Sequential patterns of emergence of autoantibodies with earlier appearance of ANA followed by anti-dsDNA, anti-RNP and -Sm antibodies have been documented in human patients (Arbuckle et al., 2003) and in murine models of SLE (Levine et al., 2006).

4.5. Molecular pathogenesis

The recognized importance of contributions of autoantibodies to molecular pathogenesis, tissue damage, subsequent disease phenotypes, and mortality pattern among SLE patients has led to elaborate evaluation of autoantibody status and disease development in animal models and in human SLE. Anti-dsDNA antibodies have been reported to be associated with glomerular damage in lupus nephritis and brain damage in neuropsychiatric lupus (Lefkowitz et al., 1996; Spatz et al., 1997; DeGiorgio et al., 2001). In an epidemiological analysis, Hitchon and Peschken (2007) reported the potential roles of anti-RNP antibodies on organ damage and anti-Sm antibodies on mortality among SLE patients. Although clinical signs and overt disease phenotypes were not evident in this study, there was a substantial elevation of autoantibody profile, documented to be a prelude to tissue injury, organ damage and disease manifestations (Arbuckle et al., 2003) and as documented by Quintana et al. (2004) autoantibody profile even among normal individuals has a predictive potential of future autoimmune disease outcome.

4.6. Possible cross- reactivity of anti-GR-MAP-8 and anti-dsDNA

Immunizations with GR-MAP-8 elevated the autoantibody response to dsDNA, whereas immunizations with BB alone did not. The highly significant correlations between anti-GR-MAP-8 antibody and anti-dsDNA autoantibody levels at 5th and 7th boosts probably reflect overlap of anti-GR-MAP-8 and anti-dsDNA antibodies. With such a highly positive correlation, it is likely that there is at least a subset of anti-GR-MAP-8 and anti-dsDNA that crossreact as was reported for mAb R4A and other anti-DNA antibodies. Preliminary results of affinity purifications of anti-dsDNA antibodies support their proposed cross-reactivity with GR-MAP-8. Anti-DNA antibodies have also been reported to cross react with epitopes on bacteria, phospholipids, and antigens on extracellular matrix (Khalil et al., 1999). ANA IFA patterns revealed very distinct and some unique immunofluorescence pattern in BB- and GR-immunized rabbits although no such differences were discernible by ANA ELISA. Differences in IFA patterns suggest variability in autoantibody responses against different autoantigens in both BB and GR groups that cannot be documented by the ANA ELISA. Even though the ages of immunized rabbits and immunizing peptides were similar, more female rabbits developed high autoantibody responses (70.9%) indicating another similarity to responses in human beings. This observation again makes the rabbit animal model similar to human SLE, where the incidence of disease is 7.5 to 9 times higher in women (Hochberg, 1997; Lopez et al., 2003). Huang et al. (2004) also found a higher gender ratio for SLE prevalence among female relatives of SLE patients.

Ballestar et al. (2007) have discussed the extremely complex nature of SLE inheritance and suggested effects of epigenetic alterations on development and progression of disease. In the rabbits studied here, pedigree analyses revealed familial or litter influences played a dominant role in leukocyte and autoantibody responses. Members of 1UA344 litter in general showed lower leukocyte and autoantibody responses. Nonresponder rabbits SM-2 and SM-4 contributed to pedigree of this full sib family. High anti-dsDNA responder litter 2YY299 and high ANA responder 1YY327 litter had high autoantibody responders SM-13 and SM-1 in their pedigree, and SM-1 also exhibited seizures (Rai et al., 2006). Familial incidence and prevalence have been documented in human SLE (Hochberg, 1997; Huang et al., 2004; Wang et al., 2007), and the present study clearly substantiates familial influence on autoantibody responses in the rabbit model. Inheritance of autoantibody responses from high responder

parents and grandparents assumes importance in the context of proposed polygenic inheritance and high heritability in human SLE and is also in agreement with the additive model in mice where genetic and environmental effects on disease phenotype are known (Mohan, 2006). The results of this study on the reproducibility of elevated autoantibody responses among progeny of high responder ancestors and the unique pattern of autoimmunity among immunized rabbits has the potential to develop rabbit models with spontaneous incidence of autoimmunity by maintaining selection pressure based on autoantibody response of parents.

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Fig. 1.

Leukocyte counts prior to immunization (WBC Pre), and responses after immunization boosts 3, 5 and 7 (WBC>3B, WBC>5B and WBC>7B). Rabbits are shown ordered according to VH1 a allotypes. After the rabbit number, asterisks indicate homozygous a1/1 rabbits, closed circles heterozygous a1/a2, and open circles homozygous a2/2 rabbits. Significance was evaluated by one-way ANOVA. Whereas prior to immunization (WBC pre) the three groups (a1/a1, a1/a2 and a2/a2) were not significantly different, for a type I error rate of 1% ($\alpha = .01$) we rejected the null hypothesis that the means are equal for all three groups (a1/a1, a1/a2 and a2/a2) for WBC3 and WBC5 (post-booster 3 and 5). For a type I error rate of 5% ($\alpha = .05$) we rejected the null hypothesis that the means were equal for all three groups post-booster 3, 5 and 7.

Fig. 2.

Results of ELISA for ANA showing OD at 450 nm in pre-immune sera (ANA pre), and in sera collected post-immunizations after 3rd, 5th and 7th boosts (ANA>3B, ANA>5B, ANA>7B). Rabbits immunized with GR-MAP-8 are grouped together followed by those immunized with BB MAP-8 backbone.

Fig. 3.

ANA immunofluorescence staining results with sera from experimental rabbits showing examples of typical (A and B panels) and some atypical patterns (C and D panels). Panels A1 to A4: pre-immune, post-3rd, -5th and -7th boost sera of rabbit BB65. Panels B1 to B4: pre-immune, post-3rd, -5th and -7th boost sera of rabbit GR62. Panels C1 and C2: GR59 pre-immune and post-5th boost sera. Panels D1 and D2: BB69 pre-immune and post-5th boost sera. Panels E1-E4 show staining with pre-immune, post-3rd, -5th and -7th boost sera of BB69 diluted 1:40.

Fig. 4.

Anti-dsDNA antibody development patterns among immunized rabbits. Results of ELISA for anti-dsDNA showing OD at 450 nm in pre-immune sera (dsDNA pre), and in sera collected post-immunizations after 3rd, 5th and 7th boosts. Rabbits immunized with GR-MAP-8 are grouped together followed by those immunized with BB MAP-8 backbone.

Fig. 5. Results of ELISA for anti-RNP (A) and anti-Sm (B), showing OD at 450 nm in pre-immune sera and in sera collected post-immunizations after 3rd, 5th and 7th boosts.

Fig. 6.

Pedigree showing key ancestors of rabbits studied here and males (large squares) and females (large circles) in the current study group. Colors in the four quadrants indicate post-immunization elevations of anti-dsDNA (blue, upper left), anti-RNP and/or anti-Sm by ELISA (yellow, lower left), ANA by IFA (red stippled, upper right) and ANA by ELISA (brown diagonal lines lower, right). The darker colors in quadrants indicate those rabbits in the present study group with highest responses as shown in Table 3. Rabbits that were only used for breeding are shown with small gray symbols. Selected key rabbits immunized in earlier studies are shown in smaller symbols with quadrants. Because their sera were tested by different ELISA, the lower right shows sera positive for ENA and/or SS-A (Ro) and/or SS-B (La) rather than positive in the ANA ELISA reported for the current animals. Two males (SM1 and GR9) exhibited seizure-like episodes and a female (GR8) exhibited nystagmus.

Table 1

Designation, sexes and allotypes of experimental rabbits

Group ID	Rabbit ID	Allotype	sex
GR 48	UA345-1	a2/2, b9k/9k	M
GR49	1UA344-1	a1/2, b9k/9k	M
GR50	1UA344-5	a1/2, b9k/9k	F
GR51	1YY119-6	a1/1, b9/9	F
GR52	1YY119-8	a1/1, b9/9	F
GR53	2YY119-6	a1/1, b9/9	M
GR54	2YY299-5	a1/1, b4/9	F
GR55	2YY299-3	a1/1, b4/9	F
GR56	UA345-2	a1/2, b4/9k	M
GR57	1UA344-2	a1/2, b5/9k	M
GR58	1UA344-6	a2/2, b5/9k	F
GR59	1YY119-7	a1/1, b9/9	F
GR60	1YY327-2	a1/1, b5/9	M
GR61	2YY327-9	a1/1, b4/5	F
GR62	2YY299-4	a1/1, b4/9	F
GR63	2YY119-8	a1/1, b9/9	F
BB64	UA345-4	a2/2, b9k/9k	F
BB65	2UA344-1	a1/2, b9k/9k	F
BB66	1UA344-3	a2/2, b5/9k	M
BB67	2YY327-8	a1/1, b4/5	M
BB68	UA345-6	a1/2, b4/9k	F
BB69	1YY327-4	a1/1, b5/9	F
BB70	1YY119-5	a1/1, b9/9	M
BB71	2YY119-7	a1/1, b9/9	M

Table 2
Leukocyte responses to immunizations among experimental rabbits^a

Rabbit ID	Group ID	WBC count in 1000s	Monocyte count	Eosinophil count	Basophil Count
IUA344-1	GR49	9.0 (9.8)	424.7 (686)	90.3 (183)	425 (450)
IUA344-2	GR57	8.8 (9.2)	322 (368)	179.3 (350)	380 (410)
IUA344-5	GR50	9.0 (10)	278.7 (302)	225.3 (312)	470 (600)
IUA344-6	GR58	11.1 (12.1)	658.3 (728)	110.7 (121)	498.3 (642)
IYY119-6	GR51	12.6 (14.6)	806.7 (1022)	252.7 (292)	445.7 (594)
IYY119-7	GR59	14.6 (19.8)	850 (990)	187.7 (252)	558.7 (630)
IYY119-8	GR52	14.5 (17.9)	1041.3 (1432)	178.3 (200)	310.7 (320)
IYY327-2	GR60	10.2 (11.6)	282 (464)	102.3 (116)	515.3 (696)
2YY119-6	GR53	15.5 (17.2)	927.3 (1058)	260.7 (344)	260 (516)
2YY119-8	GR63	11.2 (12.9)	646 (903)	220.7 (258)	224 (258)
2YY299-3	GR55	12.0 (13.1)	575.3 (917)	163.7 (262)	619 (1048)
2YY299-4	GR62	14.8 (20)	940 (1400)	197.3 (296)	434 (600)
2YY299-5	GR54	8.3 (9.5)	317.3 (438)	276 (380)	343.3 (475)
2YY327-9	GR61	14 (15.7)	801 (942)	227.7 (292)	472.7 (500)
UA345-1	GR48	11.0 (11.8)	411.3 (590)	146.7 (218)	693.7 (763)
UA345-2	GR56	9.1 (10)	340.7 (400)	156 (200)	493.3 (540)
IUA344-3	BB66	7.5 (7.8)	356.7 (546)	127.3 (171)	354.7 (390)
IYY119-5	BB70	13.2 (14.8)	639.7 (888)	159 (204)	609 (725)
IYY327-4	BB69	16.3 (17.8)	819.7 (1296)	157.3 (324)	791.3 (1782)
2UA344-1	BB65	10.7 (11.1)	427.3 (545)	320.3 (436)	516.7 (555)
2YY119-7	BB71	18.3 (20.8)	1159.3 (1248)	297.3 (356)	604.7 (656)
2YY327-8	BB67	14.9 (16)	656 (800)	198 (285)	501.7 (596)
UA345-4	BB64	10.5 (12.2)	427.3 (488)	243.7 (297)	523.3 (610)
UA345-6	BB68	11.4 (12.3)	227.3 (234)	191.7 (351)	643 (738)

^aThe mean value of the 3rd, 5th and 7th boosts is shown outside the parenthesis and the highest leukocyte response of the 3rd, 5th and 7th boosts is the value within parenthesis (WBC reference range in 1000s: 4–10; reference range/μl Monocytes; 0–300; Eosinophils, 0–100; and Basophils, 0–500). Neutrophil and lymphocyte counts remained within the reference ranges (reference range/μl Neutrophils 1,200–7,000 and Lymphocytes 1,200–7,000).

Table 3

Summary of autoantibody response among experimental rabbits^a

Rabbit ID	Group ID	GR MAP-8 ELISA	Ds DNA ELISA	Anti RNP	Anti-Sm ELISA	ANA ELISA	ANA IFA
1UA344-1	GR49	3.305 (3.031)	.49 (.305)	1.006 (.4313)	1.481 (.743)	.377 (.292)	-1+/1+/1+/+
1UA344-2	GR 57	2.935 (2.892)	2.687 (1.572)	.531 (.289)	.264 (.189)	2.581 (1.847)	1+/2+/3+/3+
1UA344-5	GR50	3.13 (2.987)	1.09 (.842)	1.036 (.767)	.502 (.342)	1.122 (.670)	-/-/-1+
1UA344-6	GR 58	2.858 (2.799)	2.937 (2.002)	.387 (.129)	1.113 (.68)	2.6885 (2.155)	-1+/1+/1+
1YY119-6	GR51	2.99 (2.873)	2.353 (1.663)	2.133 (1.544)	2.797 (2.703)	1.948 (1.204)	-1+/1+/2+
1YY119-7	GR59	2.931 (2.792)	2.857 (1.516)	1.176 (.989)	.822 (.436)	2.258 (1.757)	1+/3+/3+/3+
1YY119-8	GR52	2.868 (2.642)	.468 (.231)	.724 (.273)	1.96 (1.166)	1.115 (.773)	-/-1+/1+
1YY327-2	GR60	2.915 (2.789)	1.805 (1.213)	.413 (.308)	.567 (.318)	2.746 (2.304)	-1+/2+/2+
2YY119-6	GR53	2.942 (2.846)	1.913 (1.523)	.533 (.320)	.696 (.427)	2.26 (1.893)	-1+/3+/3+
2YY119-8	GR63	2.936 (2.883)	2.886 (2.281)	2.19 (1.722)	1.375 (1.151)	2.123 (1.720)	1+/1+/1+/1+
2YY299-3	GR55	3.275 (3.035)	2.811 (2.516)	.863 (.575)	.608 (.403)	2.467 (2.161)	-/-3+/2+
2YY299-4	GR62	2.846 (2.826)	2.845 (2.241)	.832 (.442)	.872 (.435)	1.89 (.832)	1+/4+/4+/4+
2YY299-5	GR54	2.904 (2.856)	1.865 (1.116)	2.434 (1.304)	2.569 (1.587)	1.085 (.870)	-3+/2+/3+
2YY327-9	GR61	2.903 (2.801)	.394 (.176)	.971 (.516)	.615 (.412)	1.582 (1.393)	1+/3+/4+/4+
UA345-1	GR48	3.245 (3.04)	2.647 (1.653)	1.576 (1.217)	1.283 (.846)	2.539 (1.994)	-2+/2+/3+
UA345-2	GR 56	2.943 (2.922)	2.425 (1.23)	1.31 (.734)	.2935 (2.305)	1.852 (.6517)	1+/1+/1+/2+
1UA344-3	BB66	.004 (.0013)	.522 (.26)	.421 (.245)	.253 (.155)	1.001 (.654)	-1+/2+/1+
1YY119-5	BB70	.069 (.032)	1.08 (.853)	.983 (.687)	.641 (.453)	1.275 (1.22)	-4+/2+/2+
1YY327-4	BB69	.121 (.076)	.626 (.469)	.722 (.3757)	.234 (.224)	2.217 (2.170)	3+/3+/3+/3+
2UA344-1	BB65	.734 (.473)	.492 (.316)	.627 (.473)	2.122 (1.32)	.902 (.722)	1+/3+/2+/2+
2YY119-7	BB71	.015 (.006)	.617 (.487)	.489 (.304)	.133 (.105)	2.17 (1.651)	-1+/2+/2+
2YY327-8	BB67	.08 (.03)	.299 (.178)	.614 (.401)	1.041 (.713)	.868 (.289)	-2+/2+/2+
UA345-4	BB64	.008 (.003)	.76 (.403)	1.781 (1.091)	2.14 (1.023)	1.904 (1.283)	2+/3+/2+/2+
UA345-6	BB68	.0427 (.015)	.82 (.439)	1.676 (.699)	.422 (.336)	2.651 (2.558)	-3+/3+/3+

^aNumbers shown are highest values among the sera collected after 3rd, 5th, and 7th boosts and numbers in parentheses are the means of these values. Mean antibody response OD values were: GR MAP-8, 2.88 +/- 0.11; anti-dsDNA, 1.40 +/- 0.72; RNP, 0.73 +/- 0.49; Sm 0.72 +/- 0.56, and ANA 1.38 +/- 0.66. Shaded cells indicate responder rabbits with a highest OD value 1 standard deviation above mean antibody responses. For ANA IFA immunofluorescence, scores are shown for pre- and post 3rd, 5th, and 7th boosts and shaded areas indicate those sera with at least one 3+ score.

Table 4

Correlations between leukocyte, immune, and autoantibody responses among immunized rabbits.

Leukocyte responses		Antibody, autoantibody and leukocyte responses	
1. WBC count 3rd Boost		1.WBC count at 3rd boost	
WBC at 5 th boost	0.683 **	Anti-dsDNA pre immune	0.475 *
WBC at 7 th boost	0.456 *	Anti-Sm pre immune	0.448 *
Monocyte count at 3 rd boost	0.840 **	2. WBC count at 5th boost	
Monocyte count at 5 th boost	0.528 **	Anti-dsDNA pre immune	0.617 **
Monocyte count at 7 th boost	0.435 *	ANA pre immune level	0.441 *
Eosinophil count at 3 rd boost	0.407 *	3. Monocyte Count 3rd boost	
2. WBC at 5th boost		Anti-Sm pre immune level	0.567 **
WBC count at 7 th boost	0.644 **	4. Monocyte Count 5th boost	
Monocyte count at 3 rd boost	0.554 **	Anti-dsDNA pre immune	0.505 *
Monocyte count at 5 th boost	0.740 **	5. Eosinophil count 5th boost	
Monocyte count at 7 th boost	0.720 **	ANA level 7 th boost	-0.456 *
Eosinophil count at 7 th boost	0.47 *	Anti-RNP 7 th boost	0.468 *
3. WBC count at 7th boost		Anti-Sm 7 th boost	0.405 *
Monocyte count at 5 th boost	0.485 *	6. Pre Immune Basophil Count	
Monocyte count at 7 th boost	0.837 **	ANA level 3 rd boost	-0.423 *
Basophil count at 7 th boost	0.504 *	Anti-RNP level 7 th boost	-0.487 *
4. Monocyte count at 7th boost		7. Anti-GR-MAP-8 level 3rd boost	
Pre immune monocyte count	0.418 *	Anti-dsDNA pre immune	-0.439 *
Monocyte count at 3 rd boost	0.449 *	8. Anti-GR-MAP-8 level 5th boost	
Monocyte count 5 th boost	0.488 *	Anti-dsDNA pre immune	-0.477 *
Eosinophil count 7 th boost	0.497 *	Anti-dsDNA 5 th boost	0.61 **
Basophil count at 7 th boost	0.587 **	9. Anti-GR-MAP-8 level 7th boost	
5. Eosinophil count preimmune		Anti-dsDNA pre immune	-0.46 *
Basophil count 5 th boost	-0.412 *	Anti-dsDNA 7 th boost	0.64 **
9.Eosinophil count 7th boost		10. Anti-dsDNA at 5th boost	
Basophil count 3 rd boost	-0.420 *	ANA level at 5 th boost	0.446 *
		11. Anti-dsDNA level at 7th boost	
		ANA level at 7 th boost	0.532 **
		12. Anti-RNP level 5th Boost	
		Anti-Sm level 5 th boost	0.713 **
		13. Anti-RNP level at 7th boost	
		Anti-Sm level at 7 th boost	0.591 **

* significant at 5% level

** Significant at 1% level