ASSOCIATION OF LOW C2 AND C4 SERUM LEVELS WITH THE HLA-DW2 ALLELE IN HEALTHY INDIVIDUALS*

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The complement system (C-system)[1] is one of the principal mediators of the biological effects after the antigen-antibody reaction. Up to now 18 distinct complement components have been defined including 11 acting in the classical pathway of complement activation.

Hereditary deficiencies of components of the C-system were first discovered in experimental animals (1–3). In 1960 a clinically healthy man was reported with low levels of hemolytic complement caused by a deficiency of the second component of complement (4) which could be shown to be a genetically inherited disorder (5). Subsequently, hereditary deficiencies have been described for almost all classical complement components. The hereditary deficiency reported most frequently is that of C2. Homozygous or heterozygous deficiency of C2 has been found in both healthy individuals and in association with systemic or discoid lupus erythematosus, dermatomyositis, various forms of vasculitis, anaphylactoid purpura, and rheumatic diseases (6–19). Fu et al. (16, 20, 21) and Day et al. (22) were the first to study HLA markers in families with inherited deficiencies of the C2 complement component. They found that C2 deficiency gene(s) (C2°) segregated in coupling with the haplotype HLA-A10,B18. These observations could be confirmed by others (23–26). Furthermore HLA-D typing in such families revealed a close association between the C2° gene(s) and the HLA-DW2 allele. This was first shown by Fu et al. (16, 21) and subsequently confirmed by several authors (23, 27, 28). The complete deficiency of the C4 complement component was first described by Hauptmann et al. (29) in a patient with systemic lupus erythematosus. In a family segregation study of this case, Rittner et al. (30) demonstrated a close linkage between the C4° gene(s) and the HLA-complex. The C4° gene segregated with the haplotype HLA-A2,B40,CW3; BfS, carrying hitherto an undefined HLA-D allele (RE) (31). The propositus was homozygous for the entire haplotype. In 1977 Ochs et al. (32) described in a second family the close linkage between the C4° gene(s) and HLA. In this case the C4° genes were inherited with the haplotypes HLA-A2,B12,DW2; BfS (maternal) and HLA-A2,B15,DW8; BfS (paternal).

Studies in other species showed a striking homology in the association between some complement components and the MHC. For the mouse system it could be demonstrated (33–35) that the serum protein coded for by the S-region in the H-2 complex (36) is the homologue of human C4. Furthermore Ferreira and Nussenzweig (37) showed that the functional activity of C3 is linked to H-2 during ontogeny. In 1976 Shevach et al. (38) demonstrated that a C4 deficiency gene is closely linked to the major histocompatibility complex (MHC) of the guinea pig. In analogy to man a close linkage of the Bf locus (C3-proactivator) and the MHC of the rhesus monkey has been demonstrated (39).

The close functional relationship between C2 and C4 in the classical pathway of complement

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[1] *Abbreviations used in this paper*: C, complement; C2, C4, C2, C4 deficiency gene(s); MHC, major histocompatibility complex.

activation and the gametic association between C2 deficiency and HLA-DW2 mentioned above prompted us to investigate C2 and C4 serum levels in unrelated healthy HLA-DW2 positive and negative blood donors.

Materials and Methods

Serum Samples. Serum samples were obtained by prior centrifugation of whole blood allowed to clot at room temperature for about 30 min and then at 4°C for 60 min. The sera were stored at −80°C in small volumes and were thawed only once for determinations of C2 and C4.

C2 and C4 Measurements. Serum concentrations of C4 were measured by radial immunodiffusion with commercial plates (Behring-Werke AG, Marburg/Lahn, West Germany). C2 was measured by hemolytic titration. Sheep erythrocytes were optimally sensitized with anti-sheep erythrocyte antibodies (40). EAC4 and EAC14 intermediates were prepared according to the method of Borsos and Rapp and Borsos et al., (41, 42) and Ruddy et al. (43). Before the incubation with EAC14 cells whole human sera were treated with iodine to stabilize and increase C2 hemolytic activity by using the method of Polley (44). EAC142 cells were lysed by the addition of guinea pig serum with 0.04 M EDTA (ethylene diaminotetraacetic acid) added. All titrations were done in sucrose-veronal-buffered saline with 0.03% gelatine added at an incubation temperature of 37°C. To reduce methodological variabilities all sera were titered on the same day by using the same reagents, cell intermediates, buffers, and incubation conditions. Lysis in the assays was determined spectrophotometrically from the supernatant hemoglobin at 412 nm. The extinction values were punched on paper tape and the CH50 values were calculated by a digital computer with a program that fitted linear regression lines to the experimental points.

HLA Typing. A set of more than 90 highly selected antisera was used for HLA-A and -B typing, according to the method described by Terasaki and McClelland (45). Typing for HLA-D was performed in a microculture system with 1×10^5 responding and 1×10^5 stimulating cells (R-irradiated with 2,500 rads) using established workshop homozygous typing cells. Details of this method are given in previous publications (46, 47). The mixed leukocyte culture stimulation results obtained in mean counts per minute from triplicates were recalculated in so-called stabilized relative responses, according to the method of Thomsen et al. (48) with some modifications (49).

Statistical Evaluation. To characterize the bimodality of the distributions of C2 and C4 values obtained logarithmic Gaussian distributions were optimally fitted to the experimental curves using a FORTRAN computer program (50).

Results

C2 Determinations. Hemolytic C2 complement levels were measured in 62 HLA-DW2 positive and 64 HLA-DW2 negative unrelated healthy individuals. For both the HLA-DW2 positive and the HLA-DW2 negative population bimodal distributions of C2 serum activities were obtained (Fig. 1). The mean values and ranges of the two groups in the HLA-DW2 positive and HLA-DW2 negative individuals were very similar (Table I). In addition the modal values were similar in both cases (Fig. 1) (5×10^3 and 12×10^3 U C2/ml) with a ratio of approximately 1:2. Although the shape of the curves was similar in the HLA-DW2 positive and HLA-DW2 negative population the difference of distribution of the low and high C2 titer groups was statistically significant ($\chi^2 = 8.13$, $P = 0.004$ for the χ^2 − test; the frequency values for the entries of the 2×2 table were taken from the theoretical distributions). 63% of the HLA-DW2 positive population but only 37% of the HLA-DW2 negative population had C2 levels in the low group. On the other hand only 37% of the HLA-DW2 positive population but 63% of the HLA-DW2 negative population had high C2 levels. A second bleeding of some of the individuals was taken 6 mo later to see if the results of the first bleeding were stable with time (Fig. 3). The C2 levels of five HLA-DW2 positive and five HLA-DW2 negative individuals were nearly identical at

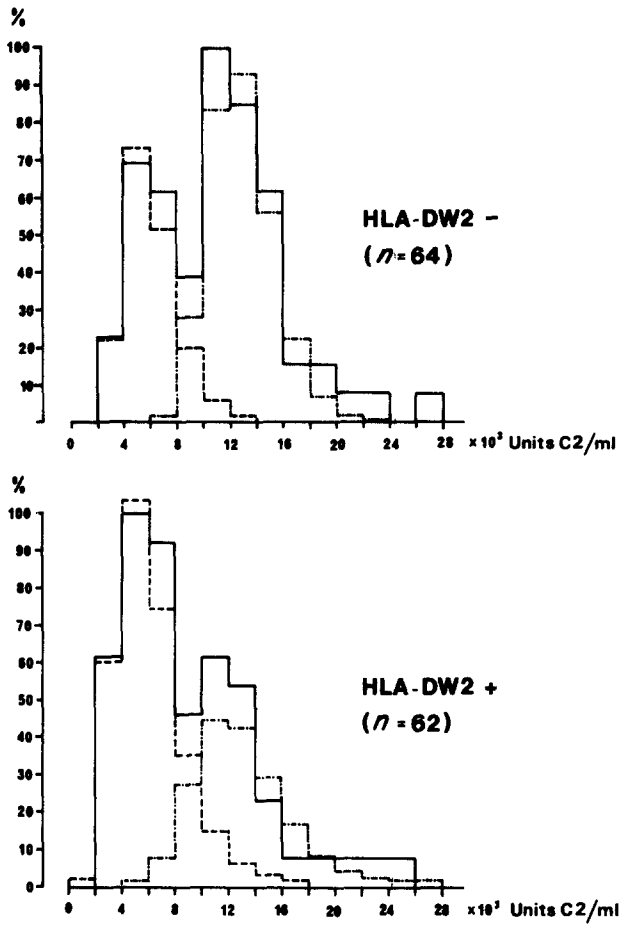

FIG. 1. Distribution of C2 serum levels in HLA-DW2 positive and negative healthy individuals. ——, experimental curves; - - -, theoretical distribution of low C2 levels; -·-·-, theoretical distribution of high C2 levels.

the second bleeding thus making it unlikely that the bimodal titer distribution curves were due to transient functional variations ($r = 0.995$, $P < 0.001$).

C4 Determinations. C4 protein was measured by immunodiffusion in 64 HLA-DW2 positive and 72 HLA-DW2 negative unrelated healthy individuals. C4 serum levels revealed in both populations a bimodal distribution although the bimodality in the HLA-DW2 negative group was not as distinct as the one of the HLA-DW2 positive group (Fig. 2). 39% of the HLA-DW2 positive population but only 15% of the HLA-DW2 negative population had low C4 protein levels whereas 61% of the HLA-DW2 positive individuals and 85% of the HLA-DW2 negative individuals had high C4 protein levels. The mean values and ranges of the C4 concentrations were similar in the HLA-DW2 positive and negative individuals (Table I). The modal values were around 30 and 42 mg/100 ml which corresponds to a ratio of 1:1.4.

Taking the same criteria as for the C2 investigation the difference in the contribution to the high and low C4 level groups between the HLA-DW2 positive and HLA-DW2 negative individuals was statistically significant ($\chi^2 = 9.84$, $P = 0.0017$). The

TABLE I

C2 and C4 Serum Levels in HLA-DW2 Positive and Negative Healthy Blood Donors

	HLA-DW2-Positive			HLA-DW2 Negative		
	n	CH50/ml serum	%	*n*	CH50/ml serum	%
Total C2	62	7,280 ± 5,138	100	64	9,340 ± 4,772	100
Low C2 individuals	39	3,530 ± 1,801	63	24	4,130 ± 1,620	37
High C2 individuals	23	11,530 ± 4,277	37	40	11,700 ± 3,747	63
		mg/100 ml			*mg/100 ml*	
Total C4	64	39.5 ± 10.2	100	72	40.4 ± 8.3	100
Low C4 individuals	25	28.6 ± 2.9	39	11	28.8 ± 3.3	15
High C4 individuals	39	46.1 ± 6.7	61	61	43.3 ± 6.5	85

results of the second bleeding 6 mo later are shown in Fig. 3; as for C2 the C4 protein concentrations in six HLA-DW2 positive and six HLA-DW2 negative individuals were stable and highly correlated to the first determination ($r = 0.996$, $P < 0.001$).

An analysis of the relationship between C2 and C4 levels in the HLA-DW2 positive population (Fig. 4) revealed a positive correlation ($r = 0.338$, $P < 0.01$).

HLA-A and -B Analysis. Allele frequencies for the HLA-A and -B series were calculated in both the HLA-DW2 positive and HLA-DW2 negative population. No gross deviations from the expected frequencies were found (i.e. increase of the HLA-B7 and B18 allele frequencies in the HLA-DW2 positive population due to the known linkage disequilibrium). This can be taken as evidence that there is no further strong association of low or high C2 and C4 levels to the HLA-A,B system.

Discussion

To our knowledge this report is the first giving information about the genetic linkage between C2 and C4 serum levels and the HLA system in healthy unrelated individuals. Previous studies on this topic dealt nearly exclusively with families where one or more members were identified as C2 or C4 homozygous deficient patients mostly affected by autoimmune or rheumatic diseases. These studies gave clear evidence that the C2 deficiency gene(s) (C2°) segregates with HLA haplotypes in families and that this deficient gene is in linkage disequilibrium with the HLA-DW2 allele, possibly with the entire haplotype HLA-A10,B18,DW2.

The genetic linkage between a C4 deficiency gene (C4°) and the HLA complex is firmly established only in two families (30, 32). From the data in the literature it seems that the deficiency of the fourth component of complement is far less frequent than the C2 deficiency. There were three reasons for us to study C2 as well C4 serum levels in HLA-DW2 positive and negative healthy individuals. First the gametic association between C2 deficiency and HLA-DW2, second the known linkage of C4 deficiency to the HLA complex, and third the close functional relationship between C2 and C4 in the activation of the complement cascade.

The results of serum C2 titers in 62 healthy unrelated HLA-DW2 positive blood donors showed a stable and clearly bimodal distribution with approximately ⅔ of the tested donors belonging to the low serum level group. We also observed in the HLA-DW2 negative population ($n = 64$) a bimodal distribution of C2 serum levels here with approximately ⅓ of the donors having low serum levels. If one compares the frequencies of low and high C2 serum levels in the HLA-DW2 positive versus the

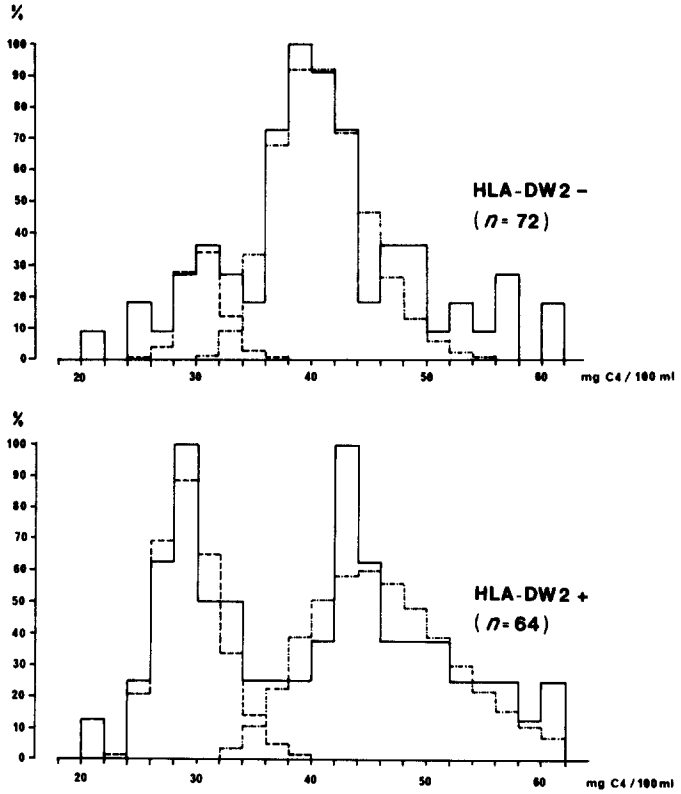

FIG. 2. Distribution of C4 serum levels in HLA-DW2 positive and negative healthy individuals. ——, experimental curves; – – –, theoretical distribution of low C4 levels; – · – · –, theoretical distribution of high C4 levels.

HLA-DW2 negative population there is a statistically significant difference showing that a preselection for the genetic trait HLA-DW2 results in a higher incidence of persons with lower C2 serum levels.

In families with C2 homozygous deficient members three categories of C2 serum levels are observed: zero (C2°/C2°), intermediate (C2°/C2), and high (C2/C2) indicating a codominant inheritance. The intermediate C2 serum levels are found in family members who have inherited one deficient and one normal C2 gene, i.e. heterozygous deficient, and high serum levels correspond to persons receiving a normal C2 gene in double dose. Based on these family data we conclude that the very distinct group of low (=intermediate) C2 serum levels in our study could represent heterozygous deficient individuals (C2°/C2). This is further supported by the observed higher incidence of intermediate C2 serum levels in the preselected HLA-DW2 positive population. A definite proof for this assumption can only be given by further family studies including the determination of the known C2 polymorphism (51–53). If the C2 deficient gene is allelic to the allotypic variants of C2, C2°/C2 persons should appear as "hemizygotes" expressing only one C2 allele.

An unexpected result was our finding that a preselection for HLA-DW2 results also in an increase of individuals having low C4 protein levels. Taking the same criteria as for the C2 study one can assume that the very distinct group with low (=intermediate)

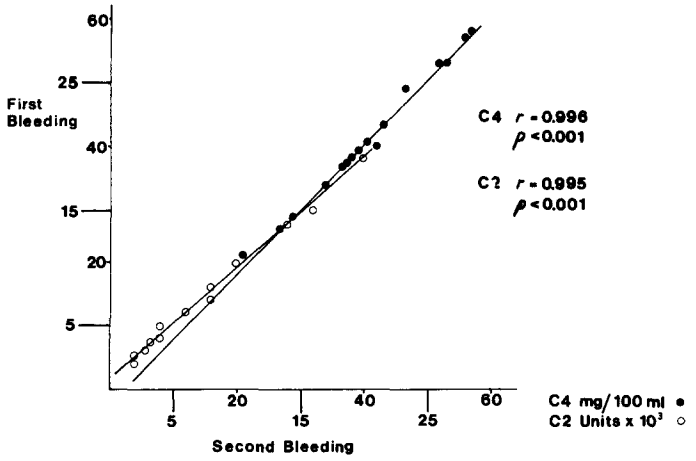

FIG. 3. Comparison of first and second bleeding of C2 and C4 levels after an interval of 6 mo.

C4 levels consists of persons carrying a C4 deficient gene. Although a genetic linkage between C4 levels and the HLA-system is clearly evident (30, 32) a formal proof for C4 deficiency in a heterozygous state cannot be given from the above data alone. As already discussed for the C2 deficient heterozygotes typing for the allotypic variants for C4 (54, 55) would give further information. A positive association of both low C2 and C4 levels with the HLA-DW2 allele is further corroborated by a statistically significant correlation coefficient (Fig. 4). If one assumes that the intermediate serum levels represent heterozygous deficiencies for C2 or C4 it is possible to estimate the frequency for C2°/C2° or C4°/C4° individuals. From our material the frequency for C2°/C2° would be 17.5%, and for C4°/C4° 3.8%. Although C2°/C2° deficiency was found in healthy individuals both values appear too high in comparison to the observed incidence of deficiencies. Since our study deals only with the association between HLA-DW2 and C2 and C4 on a phenotypical level the estimated C2°/C2° and C4°/C4° frequencies have to be taken very tentatively. Besides the C2 and C4 deficiency gene(s) associated with HLA-DW2 this apparent overestimate may be caused by other factors which are involved in the regulation of C2 and C4 serum levels. Another explanation could be the existence of an HLA-DW2 associated gene regulating the levels of both complement components. In this case our observation is not necessarily linked to C2 or C4 deficiencies. Nevertheless this would offer an interesting view on how the HLA antigen status is related to the biologically acting complement cascade. The extension of our studies could give in the future an understanding of the biological mechanisms involved in the realization of HLA associated diseases.

In summary our study in healthy unrelated individuals underlines the gametic association between C2° and HLA-DW2 which has already been established in families with various autoimmune diseases. In addition, the C4 serum levels appear to be regulated also by gene(s) associated with the HLA-DW2 allele.

These genes are possibly identical with the C2 and C4 deficiency genes known to be present on chromosome no. 6 linked to the HLA system. An alternative explanation would be the assumption of an HLA-DW2 associated gene that regulates C2 and C4 levels.

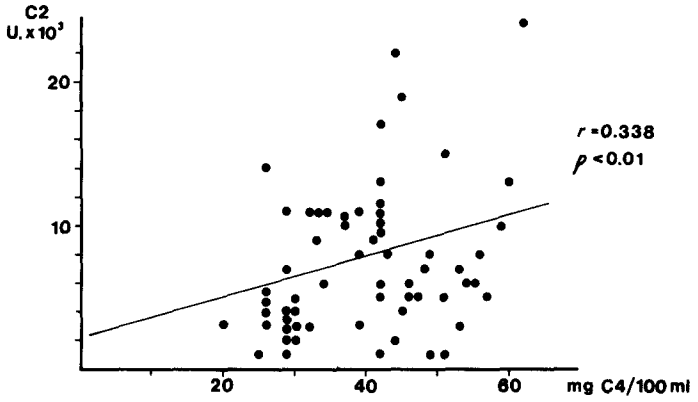

FIG. 4. Correlation of C2 and C4 serum levels in HLA-DW2 positive individuals.

As a practical consequence of this study, it is important for the clinical interpretation of complement titration data to realize that serum C2 and C4 titers are not only determined by production and consumption but also by genetic factors.

Summary

HLA typed unrelated healthy individuals (HLA-DW2 positive $n = 64$, and HLA-DW2 negative $n = 72$) were investigated for their C2 functional activity and C4 serum protein levels. For the C2 and C4 levels a bimodal distribution was found in HLA-DW2 positive and HLA-DW2 negative individuals. HLA-DW2 positive persons had a significantly higher incidence of low C2 and C4 serum levels. Our data support the concept that genes governing C2 as well as C4 serum levels are in linkage disequilibrium with the HLA-DW2 allele of the major histocompatibility complex.

HLA-A,B typing of the tested persons was performed by Doctors E. D. Albert and S. Scholz, University of Munich, Dr. Ch. Rittner, University of Bonn, and Dr. J. Bertrams, University of Essen.

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