Clin. exp. Immunol. (1981) **43,** 478–485.

Increased frequency of B8/DR3 in scleroderma and association of the haplotype with impaired cellular immune response

C. G. M. KALLENBERG, J. M. VAN DER VOORT-BEELEN, J. D'AMARO* & T. H. THE *Department of Clinical Immunology, University Hospital, Groningen, and* ** Department of Immunohaematology, University Hospital, Leiden, The Netherlands*

(Accepted for publication 26 September 1980)

SUMMARY

Twenty-eight patients with scleroderma were typed for 40 HLA antigens. A highly significant increase in the frequency of HLA-B8 and HLA-DR3 was observed, which was not related to the severity of the disease. *In vitro* lymphocyte stimulation tests were performed in 26 patients. In addition, humoral and *in vitro* cellular immune responses to a primary test antigen were measured in 12 of them. Although the group of scleroderma patients as a whole had an impaired cellular response, only the B8/DR3$^+$ patients had a strongly depressed response, whereas the immune response of the others was normal. These findings suggest an association of the haplotype B8/DR3 with impaired cellular immunity.

INTRODUCTION

Scleroderma, a connective tissue disease of as yet unknown aetiology, is associated with autoimmune phenomena. The high incidence of anti-nuclear antibodies and rheumatoid factors (Beck *et al.*, 1963; Rothfield & Rodnan, 1968; Alarcón-Segovia *et al.*, 1975) and the finding of lymphocyte cytotoxicity to cultured human target cells (Currie, Saunders & Knowles, 1971) in this disease suggest that autoimmune mechanisms are involved in its pathogenesis. In many diseases in which autoimmune phenomena are present, an association with HLA antigens B8 and to some extent Dw3 is found (Rogentine, 1976). After originally negative reports (Dausset & Hors, 1975; Crouzet *et al.*, 1975; Birnbaum *et al.*, 1977), an increased frequency of HLA-A9 (Clements, Opelz & Terasaki, 1978) and HLA-B8 (Hughes *et al.*, 1978) has been described in scleroderma. The latter association was only demonstrated in a subgroup of patients with severe systemic disease.

There is increasing evidence that immunoregulatory aberrations are primarily involved in the pathogenesis of autoimmune diseases. In mice, the immune response is controlled by genes which are strongly associated with the genes of the major histocompatibility complex. Also, in man, evidence is accumulating that immune response genes are associated with the HLA complex (De Vries *et al.*, 1977; Sasazuki *et al.*, 1978), indicating a relation between autoimmunity and HLA type.

In this report we describe the distribution of HLA antigens, including HLA-DR, in untreated scleroderma patients and their relationship to lymphocyte reactivity and primary immune response.

MATERIALS AND METHODS

Patients. Twenty-eight Dutch Caucasian patients, 13 female and 15 male, with a mean age of

Correspondence: C. G. M. Kallenberg, MD, Department of Clinical Immunology, Internal Medicine, University Hospital, Oostersingel 59, 9713 EZ Groningen, The Netherlands.

0099-9104/81/0300-0478$02.00 © 1981 Blackwell Scientific Publications

Table 1. Criteria for systemic involvement

System	Criteria	Score
Skin	Sclerosis	
	Hands	1
	Face	1
	Trunk	1
GI-tract	Radiological and/or manometric abnormalities (hypomotility)	3
Lungs	TLco < 80% predicted	1
	TLC < 80% predicted	1
	Bibasilar interstitial markings	1
Heart	Conduction abnormalities, ventricular tachycardia, multifocal PVCs on ECG	3
Kidneys	Creatinine clearance < 60 ml/min and/or malignant hypertension	3

Mild disease: score 0–4; moderate disease: score 5–8; severe disease: score > 8.

50·7 years (range 15–68 years), were investigated. The diagnosis of scleroderma was established by the presence of characteristic cutaneous lesions (in most cases biopsy-proven) involving more than just the digits. All patients underwent a standard set of diagnostic investigations: electrocardiograph, chest radiograph, pulmonary function studies (CO-transfer factor (TLco), forced expiratory volume-1 second (FEV1), vital capacity (VC) and total lung capacity (TLC)), barium swallow, urine analysis and creatinine clearance, and serological determinations (immunoglobulins, antinuclear antibody (ANA), perinuclear factor (PNF), rheumatoid factor, LE-cell phenomenon, all performed according to standard techniques). Clinical signs were assessed by two independent observers. Degree of systemic involvement was scored as shown in Table 1.

Controls. Thirty-six matched healthy volunteers (mean age $42 \cdot 4 \pm 16 \cdot 5$ years) were used as controls for lymphocyte transformation tests. Seventeen of these controls (mean age $40 \cdot 1 \pm 12 \cdot 7$ years) were immunized with the test antigen α-HPH (see below).

The incidence of HLA-A, -B, -C and -DR antigens in scleroderma patients was compared with the distribution of these antigens in a control population consisting of 123 Dutch healthy persons.

HLA typing. HLA-A, -B, and -C typing for 32 antigens was done by the NIH lymphocyte microcytotoxicity technique. HLA-DR typing was performed with the two-colour fluorescence technique as described by Van Rood, Van Leeuwen & Ploem (1976) with a few minor modifications. Testing was done for the eight officially recognized DR antigens.

Immunological methods. In vitro lymphocyte stimulation tests with mitogens and allogeneic cells were performed in 26 patients and 36 controls. Twelve patients and 17 controls were immunized with 1 mg *α-Helix pomatia* haemocyanin (HPH) subcutaneously in the deltoid region, as described by De Gast, The & Snijder (1973). Sera for antibody titre determinations were collected before and 17 days after immunization. At 17 days after immunization, venous heparinized blood was sampled for *in vitro* lymphocyte stimulation tests with the antigen.

Antibody determination. Class-specific antibodies to α-HPH were determined using an indirect ELISA technique (Weits *et al.*, 1978). Positive and negative serum pools were always included. The negative pool had a titre of <1:10. If its titre was higher than that level, the entire series was discarded. The positive pool had a titre of 1:1,280. The results were corrected for small titre variations. They are expressed as the reciprocal of the $\log_2$ of the highest dilution which still resulted in a positive reaction (1:10 taken as 1).

Lymphocyte stimulation tests. Peripheral blood mononuclear cells were obtained from fresh

heparinized blood (20 u heparin per ml of blood) by Ficoll–Isopaque density-gradient centrifugation. Cells were washed three times in RPMI with HEPES buffer (GIBCO–BIOCULT) supplemented with penicillin–streptomycin. Triplicate cultures were performed in round-bottomed microtitre plates (Cooke, U-form, 220 M-24 AR) in a final volume of 0·10 ml of the above-described medium supplemented with 25% inactivated human serum. Mitogenic stimulation was performed on 3×10^4 cells per well with PHA (Wellcome) 1 μg/ml, Con A (Calbiochem) 1 μg/ml and pokeweed mitogen (GIBCO-BIOCULT) 10 μg/ml. Antigenic and MLC stimulation was performed on 10^5 cells per well, the latter with allogeneic cells (10^5 irradiated lymphocytes from a pool of frozen lymphocytes from 10 random healthy donors). Control cultures without stimulant were included. The plates were incubated at 37°C in a humidified 5% CO_2-in-air atmosphere, 5 days for mitogenic and 6 days for antigenic stimulation. Sixteen hours before harvesting, 0·5 μCi radioactive thymidine (Radiochemical Centre, Amersham, England) was added. Thymidine uptake was determined by liquid scintillation counting. The results were expressed as disintegrations per minute (d.p.m.) per culture with stimulant minus d.p.m. of the culture without stimulant. The arithmetic mean of triplicate cultures was calculated.

Statistical methods. For HLA typing, the observed differences as well as the relative risk statistics were tested for significance by using Haldane's modification (Haldane, 1955) of Woolf's method (Woolf, 1955). The pairwise P values were corrected for the number of comparisons (40 antigens) (McDevitt & Bodmer, 1972). Differences with corrected P values $< 0{\cdot}05$ were considered significant.

Statistical analysis on lymphocyte reactivity was performed by Wilcoxon's rank sum test; P values less than 0·05 were considered significant.

RESULTS

Clinical and serological findings in 28 patients with scleroderma are summarized in Table 2. Using the criteria described in Table 1, five patients were classified as having mild disease, 15 as having moderate and eight as having severe disease.

HLA typing

Patients and controls were typed for HLA-A, -B, -C and -DR antigens. A significantly increased incidence of HLA-B8 and of HLA-DR3 was found in scleroderma patients (Table 3). B8 and DR3 were equally distributed in the subgroups with mild, moderate and severe disease (Table 4). Linkage

Table 2. Clinical and laboratory data of 28 patients with scleroderma

Raynaud's phenomenon	28	(100)*
Acrosclerosis	19	(69)
Telangiectasia	25	(89)
Arthritis	8	(29)
Pulmonary involvement	14	(50)
Gastrointestinal involvement	14	(50)
Cardiac involvement	2	(7)
Renal involvement	2	(7)
ANA (titre ≥ 1:100)	21	(75)
LE-cell phenomenon	8	(29)
PNF	2	(7)
Rheumatoid factor	8	(29)
Hyperglobulinaemia	6	(21)
Cryoglobulinaemia	1	(4)

* Figures in parentheses express results as a percentage.

Table 3. Disparate antigen frequencies in scleroderma patients compared to normal controls

Antigen	Patients	Controls	χ^2 value	P value	P corrected	Relative risk
B8	20/28	29/123	20·634	0·000057	0·0023	7·7
DR3	17/27	33/123	12·115	0·00085	0·033	4·5

Table 4. Distribution of HLA-B8 and -DR3 in subgroups of scleroderma patients

Antigens	Mild disease	Moderate disease	Severe disease
$B8^+/DR3^+$	3	9	5
$B8^+/DR3^-$	1	1	0
$B8^+$/DR n.d.	0	1	0
$B8^-/DR3^-$	1	4	3
	5	15	8

n.d. = Not determined.

Table 5. Linkage disequilibrium between B8/DR3 in patients and controls

Antigens	Controls	Patients
$B8^+/DR3^+$	23	17
$B8^+/DR3^-$	6	2
$B8^-/DR3^+$	10	0
$B8^-/DR3^-$	84	8
	123	27
Linkage disequilibrium	0·079±0·011	0·213±0·064*

* $P<0·05$.

disequilibrium between B8 and DR3 (Table 5) was almost four times higher in scleroderma patients than in the control population, suggesting that the haplotypic pair B8/DR3 is associated with the disease.

Immune response and lymphocyte reactivity

Lymphocyte reactivity on stimulation with mitogens was investigated in 26 scleroderma patients and compared with 36 matched controls. Stimulation with Con A (1 μg/ml) was significantly decreased in scleroderma ($P=0·001$), whereas lymphocyte reactivity on PHA (1 μg/ml) was not decreased ($P=0·65$, n.s.) (Fig. 1).

Twelve scleroderma patients and 17 matched healthy controls were immunized with α-HPH to test the immune response against a primary test antigen. All patients and controls did not have detectable antibodies to α-HPH before immunization. Seventeen days after immunization, class-specific antibodies and *in vitro* lymphocyte reactivity on stimulation with α-HPH were determined.

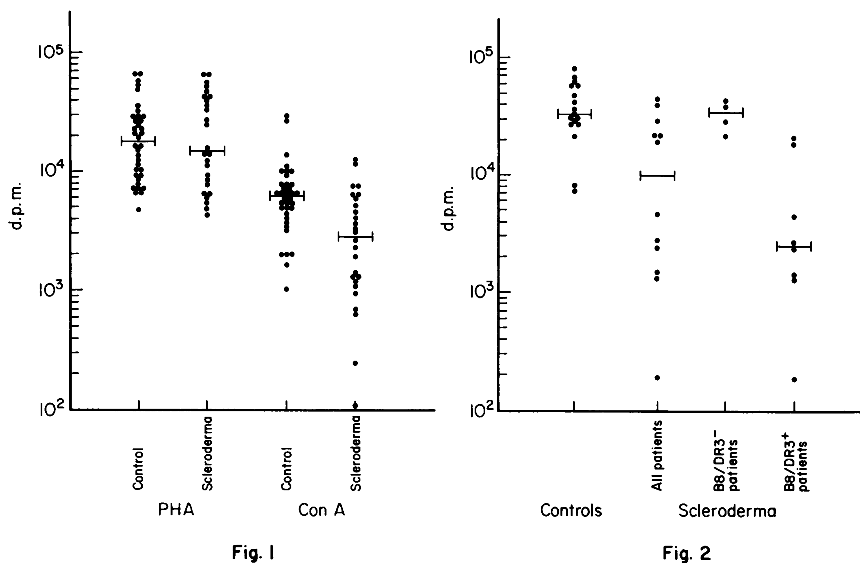

Fig. 1. *In vitro* lymphocyte reactivity to PHA, 1 μg/ml, and Con A, 1 μg/ml, in controls and scleroderma patients.

Fig. 2. *In vitro* lymphocyte reactivity to α-HPH, 15 μg/ml, in controls and scleroderma patients, related to the presence of HLA-B8/DR3.

Antibody titres in patients were not significantly different from those in controls (Table 6). However, lymphocyte stimulation *in vitro* with α-HPH, 15 and 50 μg/ml, was significantly decreased in scleroderma for both concentrations of α-HPH used (*P* values respectively 0·001 and 0·003) (see Fig. 2).

Immune response in relation to HLA

Lymphocyte reactivity on stimulation with mitogens (PHA 1 μg/ml, Con A 1 μg/ml and PWM 10 μg/ml) and with allogeneic cells was compared in B8/DR3$^+$ patients and in patients lacking both antigens. The same was done with antibody titres against α-HPH and with lymphocyte stimulation scores with α-HPH. As shown in Table 7, the *in vitro* cellular immune response to α-HPH was significantly depressed in B8/DR3$^+$ scleroderma patients, whereas the cellular response of the other patients was normal. Lymphocyte reactivity to mitogens and allogeneic cells was also obviously depressed in B8/DR3$^+$, although not statistically significant. Antibody titres were not significantly different. The observed differences in cellular immunity were not due to differences in severity of disease, as could be expected from the distribution of HLA antigens in the subgroups of the disease.

DISCUSSION

In a previous report we studied the distribution of HLA antigens in patients with Raynaud's

Table 6. Class-specific antibodies to α-HPH in scleroderma patients and controls. Results are expressed as $\log_2$ of highest positive serum dilutions (mean ± s.d.)

	Controls ($n=17$)	Patients ($n=12$)
a-αHPH-IgM	7·5 ± 1·4	7·0 ± 1·4
a-αHPH-IgA	7·7 ± 1·4	7·6 ± 0·7
a-αHPH-IgG	8·4 ± 1·5	7·7 ± 1·9

Table 7. Lymphocyte reactivity and immune response in relation to HLA in scleroderma patients

Stimulant	B8/DR3$^+$	Others	*P* value
PHA, 1 μg/ml	14·440 (16)	39·737 (10)	0·10
Con A, 1 μg/ml	2·106 (16)	4·257 (10)	0·06
PWM, 10 μg/ml	20·495 (16)	31·382 (10)	0·08
Allogeneic cells	13·803 (15)	24·330 (7)	0·3
α-HPH, 50 μg/ml	4·682 (8)	35·960 4)	0·02
α-HPH, 15 μg/ml	2·512 (8)	34·926 (4)	0·02
Antibody titres			
a-αHPH-IgM	6·8 (8)	7·5 (4)	n.s.
a-αHPH-IgA	7·9 (8)	7·3 (4)	n.s.
a-αHPH-IgG	7·9 (8)	7·3 (4)	n.s.

Results for lymphocyte reactivity are expressed as d.p.m. For each group (number of patients per group in parentheses), the median value is given. For antibody titres, results are expressed as $\log_2$ of highest positive serum dilution. Mean values are given.

phenomenon (Van der Meulen *et al.*, 1980). We demonstrated an increased frequency of HLA-B8 in those patients in whom Raynaud's phenomenon was secondary to a connective tissue disease. In the present study the patients with Raynaud's phenomenon and scleroderma were evaluated for the distribution of HLA antigens, including DR antigens, and for immune responsiveness. An association was found with B8 and DR3. Although no studies are available in which DR typing is reported in scleroderma, several former studies (Crouzet *et al.*, 1975; Birnbaum *et al.*, 1977) failed to disclose an association of HLA patterns with scleroderma, possibly because of variations in patient populations, On other studies, an increased incidence of HLA-B8 in scleroderma was reported (Rabin *et al.*, 1975; Hughes *et al.*, 1978). In the latter report the association was only observed in a subgroup of scleroderma patients with widespread visceral involvement, whereas in our study the presence of B8 and DR3 was not related to the extent of visceral disease. In addition, a strongly increased linkage disequilibrium between B8 and DR3 was observed in our patients, suggesting that the haplotypic pair B8/DR3 is associated with the disease.

The relation between this haplotype and immune responsiveness was further evaluated. A decreased cellular immune response, with a normal humoral response, was restricted to B8/DR3-positive patients, whereas immune response was normal in those patients lacking both of these antigens. In contrast to this observation a heightened immune response has been observed in B8 recipients: they reject kidney allografts more readily than others, have a better prognosis in some haematological malignancies (Falk & Osoba, 1974; Oliver *et al.*, 1977; Rogentine *et al.*, 1973), whereas the responses of healthy individuals with B8 segregate above the median response in the mixed lymphocyte reaction (Osoba & Falk, 1978). The presence of a high immune response in B8 recipients is compatible with an increased incidence of autoimmune diseases. In the animal model of these diseases, NZB and NZB/NZW F_1 mice, excessive B cell activity, due to a defect in feedback suppression (Cantor *et al.*, 1978), results in hypergammaglobulinaemia and autoantibody formation. In addition, *in vitro* studies in human systemic lupus erythematosus have demonstrated excessive B cell activity (Jasin & Ziff, 1975). In our study, B8/DR3 was associated with a decreased cellular and a normal humoral response. An impaired cellular and a normal or high humoral response was also observed in human SLE *in vitro* (Paty *et al.*, 1975; Levy *et al.*, 1970). Besides, decreased lymphocyte reactivity to mitogens was demonstrated in other autoimmune disorders (Horwitz & Garrett, 1977; Silvermann *et al.*, 1976). The discrepancy between the expected hyper-

reactivity and the observed hyporesponsiveness in our B8/DR3-positive patients might be explained by the NZB model: at 5 months of age (auto)antibody levels are high, but cellular immunity, also increased in an earlier phase of their life, is already depressed. Longitudinal studies might resolve this disparity. Besides the impaired cellular immunity, the discrepancy between humoral and cellular responses in our patients is striking, indicating that immune regulatory aberrations are present. Studies are in progress to determine suppressor cell function in this group of patients. Whatever the mechanisms, it is clear from our studies that the presence of B8/DR3 in scleroderma is associated with abnormalities in immune response.

REFERENCES

ALARCÓN-SEGOVIA, D., FISHBEIN, E., GARCIA-ORIGOZA, G. & ESTRADA-PARRA, S. (1975) Uracil-specific anti-RNA antibodies in scleroderma. *Lancet*, **i**, 363.

BECK, J.S., ANDERSON, J.R., GRAY, K.G. & ROWELL, N.R. (1963) Antinuclear and precipitating autoantibodies in progressive systemic sclerosis. *Lancet*, **ii**, 1188.

BIRNBAUM, N.S., RODMAN, G.P., RABIN, B.S. & BASSION, S. (1977) Histocompatibility antigens in progressive systemic sclerosis (scleroderma). *J. Rheumatol.* **4**, 425.

CANTOR, H., MCVAY-BOUDREAU, L., HUGENBERGER, J., NAIDORF, K., SHEN, F.W. & GERSHON, R.K. (1978) Immunoregulatory circuits among T-cell sets. II. Physiologic role of feedback inhibition *in vivo*: absence in NZB mice. *J. exp. Med.* **147**, 1116.

CLEMENTS, P.J., OPELZ, G. & TERASAKI, P.I. (1978) Association of HLA antigen A9 with progressive systemic sclerosis (scleroderma). *Tissue Antigens*, **11**, 357.

CROUZET, J., MARBACH, M.C., CAMUS, J.P., GODEAU, P., HERREMAN, G., RICHIER, D., HORS, J. & DAUSSET, J. (1975) Récherche d'une association entre antigènes HL-A et sclérodermie systémique. *Nouv. Presse Med.* **4**, 2489.

CURRIE, S., SAUNDERS, M. & KNOWLES, M. (1971) Immunological aspects of systemic sclerosis: *in vitro* activity of lymphocytes from patients with the disorder. *Br. J. Dermatol.* **84**, 400.

DAUSSET, J. & HORS, J. (1975) Some contributions of the HL-A complex to the genetics of human diseases. *Transplant. Rev.* **22**, 44.

DE GAST, G.C., THE, T.H. & SNIJDER, J.A.M. (1973) The human immune response to α-haemocyanin of *Helix Pomatia. Acta med. Scand.* **194**, 303.

DE VRIES, R.P., KREEFTENBERG, H.G., LOGGEN, H.G. & VAN ROOD, J.J. (1977) *In vitro* immune responsiveness to vaccinia virus and HLA. *N. Engl. J. Med.* **297**, 692.

FALK, J.A. & OSOBA, D. (1974) The association of the human histocompatibility system with Hodgkin's disease. *J. Immunogenet.* **1**, 53.

HALDANE, J.B.S. (1955) The estimation and significance of the logarithm of a ratio of frequencies. *Ann. hum. Genet.* **20**, 309.

HORWITZ, D.A. & GARRETT, M.A. (1977) Lymphocyte reactivity to mitogens in subjects with systemic lupus erythematosus, rheumatoid arthritis and scleroderma. *Clin. exp. Immunol.* **27**, 92.

HUGHES, P., GELSTHORPE, K., DOUGHTY, R.W., ROWELL, N.R., ROSENTHAL, F.D. & SNEDDON, I.B. (1978) The association of HLA-B8 with visceral disease in systemic sclerosis. *Clin. exp. Immunol.* **31**, 351.

JASIN, H.E. & ZIFF, M. (1975) Immunoglobulin synthesis by peripheral blood cells in systemic lupus erythematosus. *Arthritis Rheum.* **18**, 219.

LEVY, J., BARNETT, E.V., MCDONALD, N.S. & KLINENBERG, J.R. (1970) Altered immunoglobulin metabolism in systemic lupus erythematosus and rheumatoid arthritis. *J. clin. Invest.* **49**, 708.

MCDEVITT, H.O. & BODMER, W.F. (1972) Editorial. Histocompatibility antigens, immune responsiveness and susceptibility to disease. *Am. J. Med.* **52**, 1.

OLIVER, R.T.D., PILLAI, A., KLOUDA, P.T. & LAWLER, S.D. (1977) HLA linked resistance factors and survival in acute myelogenous leukemia. *Cancer*, **39**, 2337.

OSOBA, D. & FALK, J. (1978) HLA-B8 phenotype associated with an increased mixed leukocyte reaction. *J. Immunogenet.* **6**, 425.

PATY, J.G., JR, SIENKNECHT, C.W., TOWNES, A.S., HANISSIAN, A.S., MILLER, J.B. & MASI, A.T. (1975) Impaired cell-mediated immunity in systemic lupus erythematosus. *Am. J. Med.* **59**, 769.

RABIN, B.S., RODNAN, G.P., BASSION, S., GIL, T.J., III (1975) HL-A antigens in progressive systemic scleroderma. *Arthritis Rheum.* **18**, 381.

ROGENTINE, G.N., TRAPPANI, R.J., YANKEE, R.A. & HENDERSON, E.S. (1973) HL-A antigens and acute lymphocytic leukemia: the nature of the HL-A2 association. *Tissue Antigens*, **3**, 470.

ROGENTINE, G.N. (1976) HLA and disease. *Nature*, **262**, 350.

ROTHFIELD, N.F. & RODNAN, G.P. (1968) Serum antinuclear antibodies in progressive systemic sclerosis (scleroderma). *Arthritis Rheum.* **II**, 607.

SASAZUKI, T., KOHMO, Y., IWAMOTO, I., TANIMURA, M. & NAITO, S. (1978) Association between an HLA haplotype and low responsiveness to tetanus toxoid in man. *Nature*, **272**, 359.

SILVERMAN, H.A., JOHNSON, J.S., VAUGHAN, J.H. & MCGLAMORY, J.C. (1976) Altered lymphocyte reactivity in rheumatoid arthritis. *Arthritis Rheum.* **19**, 509.

VAN DER MEULEN, J., VAN DER VOORT-BEELEN, J.M., D'AMARO, J. & THE, T.H. (1980) HLA-B8 in Raynaud's phenomenon. *Tissue Antigens*, **15**, 81.

VAN ROOD, J.J., VAN LEEUWEN, A. & PLOEM, J.S.

(1976) Simultaneous detection of two cell populations by two-colour fluorescence and application to the recognition of B-cell determinants. *Nature*, **262**, 795.

WEITS, J., DE GAST, G.C., THE, T.H., ESSELINK, M.T., DEELDER, A.M., PETROVIĆ, M. & MANDEMA, E. (1978) Class-specific antibody titres (ELISA) against the primary immunogen *Helix pomatia* haemocyanin (HPH) in man. *Clin. exp. Immunol.* **32**, 443.

WOOLF, B. (1955) On estimating the relation between blood group and disease. *Ann. hum. Genet.* **19**, 251.