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Antibodies to β_2 microglobulin in the sera of patients with systemic lupus erythematosus

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Summary. The present study reports the detection of antibodies to β_2 microglobulin in the sera of patients with systemic lupus erythematosus (SLE). Using a Farr-type ammonium sulphate precipitation assay, test sera were reacted with $^{125}I\beta_2$ microglobulin, and immunoglobulins precipitated by 50% saturated ammonium sulphate. Increased β_2 microglobulin binding activity (normal values: mean ± 2 sd = 35·5 $\pm$ 7·8) was detected in 18 of 42 SLE sera. Anti-HLA sera did not reveal increased binding activity, suggesting that the antibody in SLE serum was directed toward free β_2 microglobulin. Direct validation was done by reacting $^{125}I\beta_2$ microglobulin with 4 SLE sera having increased $^{125}I\beta_2$ microglobulin binding activity, and subjecting the reactants to sucrose density gradient ultracentrifugation. Two peaks were obtained, one corresponding to free β_2 microglobulin, and the other to 7S material complexed to β_2 microglobulin. Normal sera demonstrated only one peak corresponding to unbound β_2 microglobulin. Assays of β_2 microglobulin binding activity on protein fractions obtained by Sephadex G200 column chromatography also showed the presence of increased binding activity with 7S fractions. Using a double antibody assay, the 7S material reactive to β_2 microglobulin was demonstrated to be IgG. It was also shown that sera with abnormal β_2 microglobulin binding activity had higher titres of antinuclear antibody compared to those lacking such activity ($t = 3{\cdot}18$; $P<0{\cdot}01$), indicating the pathogenetic relationship of this antibody to increased disease activity. This antibody may be responsible for some of the abnormalities of cell-mediated function previously described in SLE patients.

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INTRODUCTION

Systemic lupus erythematosus (SLE) is a disease characterized by the presence of antibodies to various nuclear and cytoplasmic constituents. Of relevance, lymphocytotoxic antibodies and antibodies to cell membrane fractions of T cells, having inhibitory activity to T-cell function have also been demonstrated (Mittal, Rossen, Sharp, Lidsky & Butler, 1970; Terasaki, Mottironi & Barnett, 1970; Stastny & Ziff, 1971; Ooi, Orlina, Pesce, Mendoza, Masaitis & Pollak, 1974; Winfield, Winchester, Wernet, Fu & Kunkel, 1975; Wernet & Kunkel, 1973). The exact stimulus for the production of these antibodies is not known, although recent evidence for a viral etiology has been produced (Lewis, Tannenberg, Smith & Schwartz, 1974). In this communication we report the detection of antibodies to β_2 microglobulin in the sera of patients with SLE, and the association of such antibodies with high titres of antinuclear antibody (ANA).

MATERIALS AND METHODS

Sera

Forty-two sera from 39 SLE patients with varying degrees of disease activity were stored at −70° until studied. The criteria for the diagnosis of SLE were those of the American Rheumatism Association (Cohen, Reynolds, Franklin, Kulka, Ropes, Shulman & Wallace, 1971). Controls consisted of laboratory personnel. Six tissue-typing antisera, directed toward the HL-A determinants A1, A2, A10, B5, B7, and B8, obtained from the National Institutes of Health and made available through the courtesy of Dr Paul Nathan, Tissue Typing Laboratories, University of Cincinnati, were also studied.

Assay for $^{125}I\beta_2$ microglobulin binding activity

This was done by two techniques: (a) ammonium sulphate precipitation assay; (b) double antibody assay.

(a) *Ammonium sulphate precipitation assay.* This was used as a screening procedure for the detection of increased β_2 microglobulin binding activity in SLE sera; sera with such activity were then tested by more definitive procedures as described below. Lyophilized $^{125}I\beta_2$ microglobulin (Pharmacia Laboratories, Piscataway, New Jersey) with a specific activity of 0·094 μCi/ng was reconstituted with distilled water to give a solution with a concentration of 6 ng/ml. $^{125}I\beta_2$ microglobulin was characterized as a single band on polyacrylamide gel electrophoresis, and as a single symmetrical peak by sucrose density gradient ultracentrifugation. About 80–90% of its radioactivity was precipitated in the binding assay by three different antisera to β_2 microglobulin. One antiserum was a generous gift from Dr P. W. Hall, Cleveland Metropolitan Hospital; the second was commercial goat antiserum to human β_2 microglobulin (Kallestad Laboratories, Chaska, Minnesota); the third, Serum 444, was derived from our laboratory by immunization of a rabbit with purified β_2 microglobulin isolated from the urine of post-renal transplantation patients.

The procedure of the binding assay was as follows: 100 μl of test serum, previously inactivated by heating at 56° for 30 min was added to 12 × 75 mm propylene test tubes and diluted with 0·85 ml of phosphate buffered saline (PBS). An aliquot of 0·05 ml of $^{125}I\beta_2$ microglobulin was then added and the mixture incubated at 37° for 30 min followed by overnight incubation for 16 h at 4°. One ml saturated ammonium sulphate was added and mixing done in a rotator at 4° for 30 min. The tube was centrifuged at 1000 *g* for 60 min. One ml of supernatant (S fraction) was carefully transferred off, leaving precipitate and the remainder of the supernatant (R fraction). Radioactivity of both fractions was determined in an automatic gamma counter, and per cent in the precipitate calculated according to the formula:

$$\frac{R-S}{R+S} \times 100 = \text{percent } \beta_2 \text{ microglobulin in precipitate.}$$

Fourteen replicates on a single sample were done; the coefficient of variation of the assay was 5·1%.

(b) *Double antibody assay.* By this method the soluble $^{125}I\beta_2$ microglobulin-anti β_2 microglobulin complexes were precipitated by goat anti-human IgG. Anti-human IgG was chosen because of the sedimentation characteristics of material complexing to β_2 microglobulin as demonstrated by sucrose density gradient ultracentrifugation. (See below.) The precipitating capacity of the goat anti-human IgG (Meloy Laboratories, Inc., Springfield, Virginia) was determined initially, and a ratio of 40:1 of antibody: antigen was found to be required to ensure complete precipitation of IgG in serum. The actual procedure consisted of the following steps: An aliquot of 0·02 ml of test serum was diluted to 0·2 ml with PBS followed by addition of 0·01 ml $^{125}I\beta_2$ microglobulin. After the incubation period of 30 min at 37° followed by 16 h at 4°, 0·8 ml goat anti-human IgG was added. Thereafter the remainder of the assay, including the calculations, was done under identical conditions as for the ammonium sulphate precipitation procedure. This assay was performed on 15 normal sera and 7 SLE sera.

Sucrose density gradient ultracentrifugation analysis of reactants

Four sera from SLE patients with precipitated counts of 58, 56, 54 and 53%, and 2 control sera with precipitated counts of 35 and 38% as determined by the ammonium sulphate precipitation assay were further analyzed in the following manner:

One hundred microlitres of heat inactivated test serum, diluted with 0·85 ml PBS was reacted with 50 μl of $^{125}I\beta_2$ microglobulin. The mixture was incubated at 30 min followed by 16 h incubation at 4°. An aliquot of 0·2 ml of the reactants was then

layered onto a 10–40% sucrose gradient in veronal buffered saline (0·005 M veronal, 0·145 M saline pH 7·4) and centrifuged at 35,000 rev/min for 15 h at 4° in an L 2 Spinco model with an SW 50.1 rotor. Fractions of 0·4 ml were collected from the bottom of the gradient and radioactivity of each fraction quantified in a Packard Model automatic gamma counter (Packard Instruments, Inc., Downers Grove, Illinois). Markers were human serum albumin (HSA 4·6S), beef catalase (11·2S), and human IgG (7S) and IgM (19S). HSA, IgM and IgG were measured by radial immunodiffusion and catalase by the disappearance of peroxide as measured spectrophotometrically at 240 nm (Beers & Sizer, 1952).

Isolation of β_2 microglobulin and production of antiserum (444) *to β_2 microglobulin*

Twelve litres of urine from post-renal transplant patients were pooled and concentrated by ultracentrifugation using the hollow fibre artificial kidney (Cordis Corporation, Miami, Florida) with a molecular cut off of about 5000 mol. wt. The retained portion was then filtered by ultrafiltration using a PM-30 membrane, which passes molecules of less than 25,000 mol. wt. and this filtrate was reconcentrated with a UM-2 membrane. The portion retained by the UM-2 membrane was fractionated by elution with PBS through a Sephadex G50 column (6 × 50 cm). The protein concentration of the fractions was obtained by monitoring the absorbancy at 280 nm and the fractions tested for β_2 microglobulin using the Ouchterlony double diffusion procedure with the antiserum supplied by Dr Hall. Positive fractions were pooled, concentrated with a UM-2 membrane and material of molecular weight less than 5000 removed by passage through a Sephadex G25 column (2·5 × 25 cm). Fractions reacting with anti-β_2 microglobulin were pooled, concentrated with a UM-2 membrane and purified by isoelectric focusing using an LKB 8101 column (LKB Instruments, Rockville, Maryland) utilizing carrier ampholytes (pH 5–6·5), and allowing the gradient to develop for 48 h at 4° and 500 V (Haglund, 1971). Fractions were collected and absorbance at 280 nm monitored in each. A single peak of protein was recovered (pI 5·5) which reacted with anti-β_2 microglobulin. A rabbit was immunized with the purified β_2 microglobulin. An antiserum (444) was derived which gave a reaction of total identity with the Hall antiserum on Ouchterlony plates against purified β_2 microglobulin.

Titration of antinuclear antibody (ANA)

This was done by the technique previously described (Ooi *et al.*, 1974; Pollak, 1964). Modifications include the use of Sprague-Dawley rat liver as a substrate, and a fluoresceinated anti-human gamma globulin conjugate with an F/P molar ratio of 2:3.

Fractionation of sera

Three sera with abnormal β_2 microglobulin binding activity and characterized to have 7S material complexing with $^{125}I\beta_2$ microglobulin were fractionated by applying 1 ml of each serum to a 2·5 × 100 cm Sephadex G200 column. The proteins were eluted with 0·1 M Tris HCl pH 8 and 0·2 M NaCl buffer. Protein content of eluates was monitored by their absorbancy at 280 nm and the position of IgM, IgG, and albumin monitored by Ouchterlony double diffusion methods. The protein fractions were concentrated to their original volume by ultrafiltration using an Amicon XM-50 membrane, and tested for β_2 microglobulin binding activity by the ammonium sulphate precipitation assay.

RESULTS

The results of the $^{125}I\beta_2$ microglobulin binding assay by the ammonium sulphate precipitation method are shown in Fig. 1. The mean binding of 38 normal sera was 35·5 %, with a standard deviation of 3·9%. A binding value of 43·3%, greater than two standard deviations from the mean was considered indicative of increased binding activity. Eighteen of 42 SLE sera studied showed increased binding activity. Three monospecific antisera to β_2 microglobulin gave binding values of 80, 86 and 90%. Anti-HLA sera did not reveal abnormal binding activity.

By the double antibody assay, 15 normal sera showed 0% $^{125}I\beta_2$ microglobulin binding activity. Seven SLE sera, selected for their high β_2 microglobulin binding activity as demonstrated by the ammonium sulphate precipitation method (ranging from 47 to 56%) when tested by the double antibody assay, exhibited abnormal binding values ranging from 5 to 13% (Table 1).

To demonstrate directly the presence of material complexing to $^{125}I\beta_2$ microglobulin, test sera were reacted with $^{125}I\beta_2$ microglobulin, and analysis of the reactants done by sucrose density gradient ultracentrifugation; this procedure was done with 4 SLE

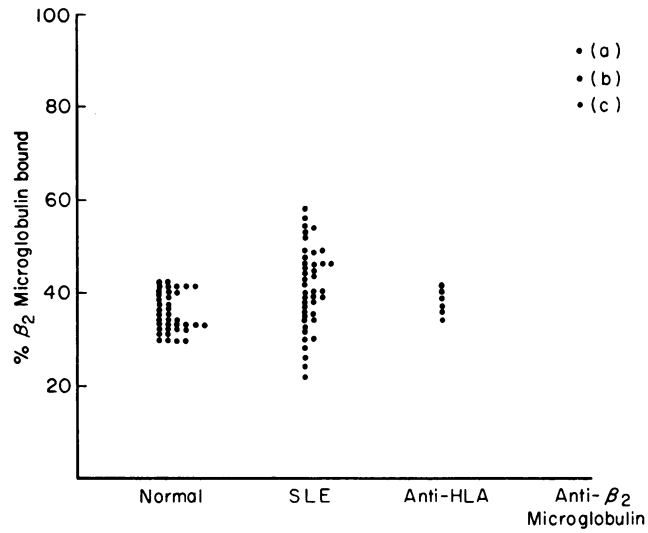

Figure 1. β_2 Microglobulin binding activity of normal, SLE, anti-HLA and specific anti-β_2 microglobulin sera as measured by the ammonium sulphate precipitation method. (A = serum 444, rabbit anti-β_2 microglobulin derived in our laboratory; B = Hall rabbit anti-human β_2 microglobulin serum; C = Kallestad goat anti-human β_2 microglobulin serum). Binding values of normal sera are 35·5 ± 7·8 (mean ± 2 sd). Eighteen of 42 SLE sera, and specific anti-β_2 microglobulin sera demonstrated increased β_2 microglobulin binding activity. Anti-HLA sera showed binding activity within normal values.

sera and 2 normal sera. The results of one fractionation procedure are shown in Fig. 2. Two peaks of radioactivity were obtained, one corresponding to unbound β_2 microglobulin, and the other to 7S material complexed to β_2 microglobulin; similar results were obtained with all 4 SLE sera. When this procedure was done with normal sera, only one peak, corresponding to β_2 microglobulin was seen.

The results of the ammonium sulphate precipitation assay for $^{125}I\beta_2$ microglobulin binding activity of different protein fractions separated by Sephadex G200 column chromatography are shown in Table 1. It demonstrates the presence of increased binding activity in the 7S fractions.

The relationship between β_2 microglobulin binding activity and titres of ANA are shown in Fig. 3.

Table 1. β_2 Microglobulin binding activity of normal and SLE sera and of their fractions as determined by the ammonium sulphate precipitation method and by the double antibody assay

		% β_2 Microglobulin binding activity			
Type of serum (fraction)	Number tested	Double antibody assay	Ammonium sulphate precipitation method		
Normal (whole)	15	0	25–40		
SLE (whole)	7	5–13	47–56		
Normal (7S)	3	NT	(a) 22	(b) 20	(c) 30
Normal (19S)	3	NT	(a) 16	(b) 15	(c) 24
Normal (albumin)	3	NT	(a) 0	(b) 0	(c) 0
SLE (7S)	3	NT	(a) 35	(b) 53	(c) 40
SLE (19S)	3	NT	(a) 12	(b) 22	(c) 16
SLE (albumin)	3	NT	(a) 0	(b) 0	(c) 0

NT, not tested.

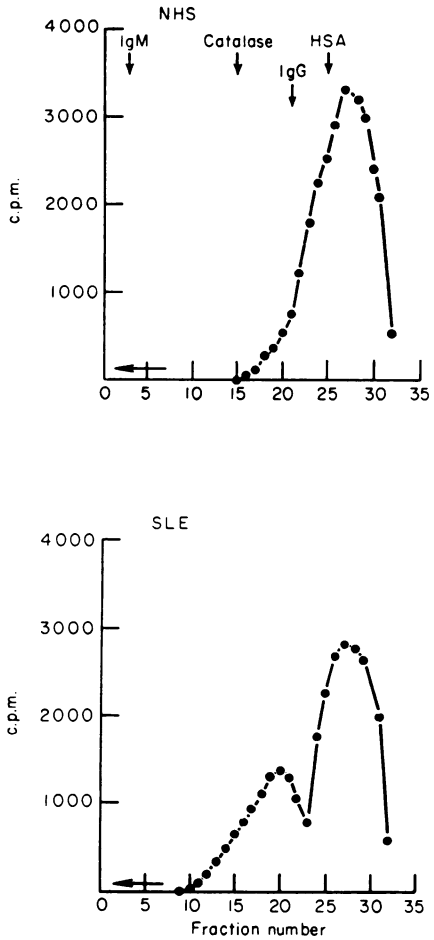

Figure 2. Sucrose density ultracentrifugation of normal serum (top panel) and of SLE serum (bottom panel) reacted with $^{125}I\beta_2$ microglobulin. Markers were human serum albumin (HSA), IgG, IgM, and catalase. With normal serum, one peak (β_2 microglobulin) was observed. With SLE serum, two peaks developed, one corresponding to unbound β_2 microglobulin and the other to 7S material complexed to β_2 microglobulin.

Sera with abnormal β_2 microglobulin binding activity had higher titres of ANA (geometric mean of reciprocal titre 34·6) compared to those with binding activity within normal limits (geometric mean of reciprocal titre 4·5); the differences between the geometric means of the two groups were statistically significant ($t = 3{\cdot}18$; $P<0{\cdot}01$).

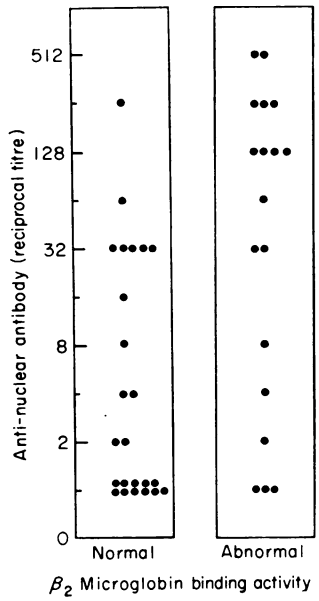

Figure 3. Relationship between reciprocal titres of ANA and β_2 microglobulin binding activity of SLE sera. The differences between the mean geometric titres of the two groups are statistically significant ($t = 3{\cdot}18$; $P<0{\cdot}01$).

DISCUSSION

β_2 Microglobulin is a low molecular weight protein (mol. wt 11,800) originally isolated from the urine of patients with tubular proteinuria (Berggard & Bearn, 1968). It has since been found to have a primary structure with a high degree of homology with the constant domain of human IgG (Smithies & Poulik, 1972; Peterson, Cunningham, Berggard & Edelman, 1972), and to be a subunit of purified HLA preparations (Grey, Kubo, Colon, Poulik, Cresswell, Springle, Turner & Strominger, 1973; Tanigaki, Nakamuro, Appella, Poulik & Pressman, 1973; Peterson, Rask & Lindblom, 1974). It is present on the cell surface of leucocytes and is synthesized by normal lymphocytes and other cell lines (Nilsson, Evrin & Welsh, 1974). It is not certain if β_2 microglobulin exists as a free form or if it is inevitably associated as an HLA antigen. Recent description of two isomorphous forms of β_2 microglobulin with different isoelectric points, has suggested that it may exist in both forms (Ricanati, Hall & Vacca, 1975).

The present study demonstrates the presence of anti-β_2 microglobulin in the sera of patients with SLE by several methods. By the ammonium sulphate precipitation method, a significant proportion of SLE sera are found to have abnormal β_2 microglobulin binding activity. Sucrose density gradient

ultracentrifugation demonstrates the presence of 7S material complexing to $^{125}I\beta_2$ microglobulin. Studies on protein fractions obtained by column chromatography also reveal the 7S fractions to contain abnormal binding activity. The double antibody assay demonstrates that this 7S material is probably IgG.

The absence of any significant binding activity of anti-HLA sera to β_2 microglobulin would suggest that the antibody is directed toward 'free' β_2 microglobulin. This lack of reactivity of antisera against HL-A with free β_2 microglobulin has previously been reported (Peterson *et al.*, 1974). Likewise, antibodies which strongly bind to free microglobulin have been shown to react weakly, if at all, with β_2 microglobulin attached to the large HL-A polypeptide chain (Peterson *et al.*, 1974).

The positive correlation between abnormal β_2 microglobulin activity and high titres of ANA suggests a significant pathogenetic relationship of anti-β_2 microglobulin to the increased disease activity of SLE.

The finding of anti-β_2 microglobulin in the sera of patients with SLE has several implications. Anti-β_2 microglobulin produced by the immunization of animals with β_2 microglobulin has been shown to inhibit T-cell function as evidenced by its ability to inhibit the mixed lymphocyte reaction, and to depress PPD-induced proliferation of sensitized lymphocytes (McCalmon, Kubo & Grey, 1975). Similar abnormalities of cell-mediated function have been documented to occur in patients with SLE. In particular, serum factors in SLE have been described which inhibit the response of lymphocytes to PHA and to stimulation by allogeneic cells (Cousar & Horwitz, 1973; Williams, Lies & Messner, 1973; Horwitz, 1972). This factor was detected in the immunoglobulin G rich fraction of the serum by Cousar & Horwitz (1973) and by Williams *et al.* (1973). In some instances, the presence of anti-β_2 microglobulin in SLE serum may be responsible for these abnormalities. In addition, Wernet & Kunkel (1973) have shown that SLE sera contain antibodies directed against different lymphocyte components, some of which interfered with HLA typing, and also depressed T-cell function.

The exact stimulus for the formation of anti-β_2 microglobulin is not known. Concordant with the current hypothesis that SLE is due to a virus infection, it is attractive to speculate that virus transformed cells, which have been shown to have different cell surface characteristics (Mintz & Sachs, 1975), contain abnormal forms of β_2 microglobulin which elicit this response. An alternative explanation, which would better explain the relationship between the presence of abnormal β_2 microglobulin binding activity and high titres of ANA (used as an indicator of disease activity) would be that significant tissue injury gives rise to altered forms of β_2 microglobulin leading to the antibody response.

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REFERENCES

Beers R.F.Jr & Sizer I.W. (1952) A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *J. Biol. Chem.* **195,** 133.

Berggard I. & Bearn A.G. (1968) Isolation and properties of a low molecular weight β_2-globulin occurring in human biological fluids. *J. Biol. Chem.* **243,** 4095.

Cohen A.S., Reynolds W.E., Franklin E.C., Kulka J.P., Ropes M.W., Shulman L.E. & Wallace S.L. (1971) Preliminary criteria for the classification of systemic lupus erythematosus. *Bull. Rheum. Dis.* **21,** 643.

Cousar J.B. & Horwitz D.A. (1973) Inhibition of lymphocyte activation by sera of patients with systemic lupus erythematosus. *Arth. Rheum.* **16,** 539 (Abstract).

Grey H.M., Kubo R.T., Colon S.M., Poulik M.D. Cresswell P., Springle T., Turner M. & Strominger J.L. (1973) The small subunit of HL-A antigen is β_2 microglobulin. *J. exp. Med.* **138,** 1608.

Haglund H. (1971) Isoelectric focusing in pH gradients—A technique of fractionation and characterization of ampholytes. *Methods of Biochemical Analysis* (ed. by D. Glick), p. 1. Interscience Press, New York.

Horwitz D.A. (1972) Impaired delayed hypersensitivity in systemic lupus erythematosus. *Arth. Rheum.* **15,** 353.

Lewis R.M., Tannenberg W., Smith C. & Schwartz R.S. (1974) C-type viruses in systemic lupus erythematosus. *Nature,* **252,** 78.

McCalmon R.T., Kubo R.T. & Grey H.M. (1975) Effect of anti-β_2 microglobulin on antigen and allogeneic lymphocyte induced proliferation of human lymphocytes. *J. Immunol.* **114,** 1766.

Mintz U. & Sachs L. (1975) Membrane differences in peripheral blood lymphocytes from patients with CLL and Hodgkins disease. *Proc. Nat. Acad. Sci.* **72,** 2428.

Mittal K.K., Rossen R.D., Sharp J.T., Lidsky M.D. & Butler W.T. (1970) Lymphocyte cytotoxic antibodies in systemic lupus erythematosus. *Nature,* **225,** 1255.

NILSSON K., EVRIN P.E. & WELSH K.I. (1974) Production of β_2 microglobulin by normal and malignant human cell lines and peripheral lymphocytes. *Transplant. Rev.* **21**, 53.

OOI B.S., ORLINA A.R., PESCE A.J., MENDOZA N., MASAITIS L. & POLLAK V.E. (1974) Lymphocytotoxic antibodies in patients with systemic lupus erythematosus. *Clin. exp. Immunol.* **17**, 237.

PETERSON P.A., CUNNINGHAM B.A., BERGGARD I. & EDELMAN G.M. (1972) β_2 microglobulin—a free immunoglobulin domain. *Proc. Nat. Acad. Sci.* **69**, 1697.

PETERSON P.A., RASK L. & LINDBLOM J.B. (1974) Highly purified papain-solubilized HL-A antigen contains β_2-microglobulin. *Proc. Nat. Acad. Sci.* **71**, 35.

POLLAK V.E. (1964) Antinuclear antibodies in families of patients with systemic lupus erythematosus. *New Eng. J. Med.* **271**, 165.

RICANATI E., HALL P.W. & VACCA C. (1975) The demonstration and characterization of isoproteins of beta$_2$ microglobulin. *Clin. Res.* **21**, 530A (Abstract).

SMITHIES O. & POULIK M.D. (1972) Initiation of protein synthesis at an unusual position in an immunoglobulin gene. *Science*, **175**, 187.

STASTNY P. & ZIFF M. (1971) Antibodies against cell membrane constituents in systemic lupus erythematosus and related diseases. *Clin. exp. Immunol.* **8**, 543.

TANIGAKI N., NAKAMURO K., APPELLA E., POULIK M.D. & PRESSMAN D. (1973) Identity of the HL-A common protein fragment and β_2 microglobulin. *Biochem. Biophys. Res. Commun.* **55**, 1234.

TERASAKI P.I., MOTTIRONI V.E. & BARNETT E.V. (1970) Cytotoxins in disease. Autocytotoxins in lupus. *New Eng. J. Med.* **283**, 724.

WERNET P. & KUNKEL H.G. (1973) Demonstration of specific T-lymphocyte membrane antigens associated with antibodies inhibiting the mixed leucocyte culture in man. *Transplant. Proc.* **5**, 1875.

WILLIAMS R.C. JR, LIES R.B. & MESSNER R.P. (1973) Inhibition of mixed leucocyte culture responses by serum and γ-globulin fractions from certain patients with connective tissue disorders. *Arth. Rheum.* **16**, 597.

WINFIELD J.B., WINCHESTER R.J., WERNET P., FU S.M. & KUNKEL H.G. (1975) Nature of cold-reactive antibodies to lymphocyte surface determinant in systemic lupus erythematosus. *Arth. Rheum.* **18**, 1.