Clin. exp. Immunol. (1973) **13**, 603–611.

STUDIES ON PREGNANCY-ASSOCIATED GLOBULIN

C. H. W. HORNE, A. L. C. McLAY, H. B. TAVADIA, I. CARMICHAEL, ANNETTE C. MALLINSON, A. A. C. YEUNG LAIWAH, MAIR A. THOMAS AND R. N. M. MacSWEEN

University of Glasgow, Department of Pathology and Medical Division, Royal Infirmary, Glasgow G4 0SF; and the Department of Pathology, Western Infirmary, Glasgow G11 6NT

(*Received* 26 *September* 1972)

SUMMARY

Using a radial immunodiffusion technique the serum levels of pregnancy-associated globulin (PAG) have been measured in eighteen females before and during administration of combined oestrogen/progestogen oral contraceptives, in twenty-eight females before and after the oestrogen or progestogen component of oral contraceptives and in ten males before and during stilboestrol therapy for prostatic carcinoma. Significant increases in PAG levels were observed during administration of (1) combined oestrogen/progestogen oral contraceptives, (2) the oestrogen component of the oral contraceptive and (3) stilboestrol. No change was observed after administration of the progestogen component.

The serum PAG levels have also been determined in seventy-three patients with primary biliary cirrhosis, seventy-three hospital in-patients and forty-six patients with various malignant diseases. A marked increase in the percentage of PAG positive sera was observed in all disease groups, particularly in malignant disease. However, compared with late pregnancy, the serum levels in all three groups were low. Preliminary experiments have failed to show that this globulin possesses immunosuppressive properties.

INTRODUCTION

There is now a considerable body of evidence to suggest that serum α_2-globulins both from normal and pathological sera can depress immune responses as assessed by skin allograft survival (Mannick & Schmid, 1967), lymphocytic responsiveness to phytohaemagglutinin stimulation (Cooperband *et al.*, 1968) and electrophoretic mobility of macrophages (Field & Caspary, 1971). Recent work has also shown that in pregnancy there is a plasma factor which can suppress the reactivity of mixed leucocyte cultures (Kasakura, 1971), and in addition Smith, Caspary & Field (1972) have described in pregnancy a serum factor which depresses lymphocytic responsiveness to purified protein derivative (PPD). The suggestion by Stimson (1972) that a serum macroglobulin present in pregnancy sera and sera from

Correspondence: Dr C. H. W. Horne, Department of Pathology, Aberdeen Royal Infirmary, Aberdeen.

patients with malignant disease may be an immunosuppressive globulin has prompted us to describe our own findings concerning a little known serum α_2-macroglobulin (MacLaren & Alper, personal communication), sometimes referred to as pregnancy associated globulin (PAG). This globulin, originally thought to be peculiar to pregnancy, has been shown to be present in 18% of sera from normal males and females (MacLaren *et al.*, 1966). It has also been found in the sera of a series of women taking combined oestrogen/progestogen oral contraceptives (MacLaren *et al.*, 1966). There are, however, no studies to show whether the oestrogen or progestogen component of such preparations is responsible for increases in serum PAG levels, nor have serum PAG levels been estimated in disease processes.

We report here the serum levels of PAG, as determined by radial immunodiffusion assay, (1) in women before and during administration of combined oestrogen/progestogen oral contraceptives, (2) in normal females taking either the oestrogen or the progestogen component of an oral contraceptive, (3) in males before and during stilboestrol therapy for prostatic carcinoma, (4) in patients with primary biliary cirrhosis and in age/sex matched hospital in-patients and (5) in patients with malignant disease. Preliminary attempts to demonstrate an immunosuppressive role for PAG are also reported.

MATERIALS AND METHODS

Serum samples, which were stored at −20°C, were obtained from the following groups of subjects.

Combined oestrogen/progestogen-treated females

A group of eighteen healthy females attending a Family Planning Clinic (mean age 23·8 years, range 18–47) who were given combined oestrogen/progestogen oral contraceptives. Six received Ovulen-100, four received Volidan and eight received Norinyl (mean duration of treatment 9·3 weeks, range 2–26 weeks). Serum samples were obtained before and during administration of these preparations.

Oestrogen and progestogen-treated females

Two groups of fourteen healthy females (mean ages 20·6 and 20·9 years, ranges 17–28 and 17–30) who were given respectively either 50 μg of mestranol (oestrogen) or 1 mg ethynodiol diacetate (progestogen) daily for a 3-week period beginning on day 5 of the menstrual cycle. Serum samples were obtained on day 3 of the cycle and once per week during treatment and for the 3 subsequent weeks. All of these subjects have been included in a previous study (Horne *et al.*, 1971).

Stilboestrol-treated males

Ten males subjects (mean age 68·9 years, range 58–78) with histologically proven prostatic carcinoma who were treated with stilboestrol (25 mg b.d. for first 3 months, then 25 mg/day for further 3 months) or Honvan (given i.v. 250 mg day 1, 500 mg i.v. day 2, 1 g i.v. days 3–7, then stilboestrol as previously detailed). The main duration of treatment was 8·6 weeks, range 1–16 weeks. Serum samples were obtained before and during therapy.

Normal pregnancy

A group of thirty-seven normal pregnant patients (mean age 27·5 years, range 18–41;

mean duration of pregnancy 37·3 weeks, range 30–41 weeks). Most of these patients have been included in a previous study (Horne, Howie & Goudie, 1970).

Primary biliary cirrhosis and hospital in-patients

(1) A group of sixty-seven females and six male patients (mean age 57·8 years, range 41–79) who had clinical, biochemical and serological findings consistent with the diagnosis of primary biliary cirrhosis (Goudie, MacSween & Goldberg, 1966; Scheuer, 1967; Sherlock, 1968). In twenty-seven cases liver biopsy evidence of this disease was also available. All of these subjects had been included in a previous study (MacSween *et al.*, 1972).

Serum was also obtained from age/sex matched hospital inpatients who had a variety of diseases (joint disease—osteoarthritis, psoriatic arthropathy, thirty-one; non-toxic goitre, twenty-one; miscellaneous skin diseases, five; neurological and/or muscular disorders, five; diabetes mellitus, four; miscellaneous group, seven). Both test and control sera were obtained from the files of the regional diagnostic immunopathology laboratory, care being taken to ensure that the test and its control serum had been stored at $-20°C$ for a similar period ± 2 months.

(2) A further group of twelve primary biliary cirrhosis patients previously shown to have serum factors inhibitory to lymphocyte transformation induced by PHA and PPD (MacSween *et al.*, in preparation).

Malignant disease

Twenty-four male, twenty-two female hospital in-patients (mean age 55·6 years, range 20–85) who had a variety of malignant diseases (malignant lymphoid neoplasms, fourteen; carcinoma of gastrointestinal tract, fourteen; carcinoma of bronchus, twelve; carcinoma of breast, three; carcinoma of female genital tract, three). Of these 33% were untreated.

Serum PAG assay

Sera were screened for the presence of PAG by double immunodiffusion in agar gel at room temperature, and serum PAG concentration was measured on duplicate specimens by radial immunodiffusion, using a specific rabbit antiserum (Fahey & McKelvey, 1965; Mancini, Carbonara & Heremans, 1965). Antiserum to PAG was prepared by immunizing rabbits with whole pregnant human serum in Freund's complete adjuvant, the resultant antiserum being absorbed with a normal serum known not to contain this protein. The antiserum obtained was monospecific and gave a reaction of complete identity with an antiserum provided by Dr C. A. Alper, Boston, U.S.A., when tested against pregnant human serum. Multiple sera from any one subject, and paired test and control sera, were always tested on the same assay plate to minimize interplate variation (Thompson *et al.*, 1969), and concentration was expressed as a percentage of a standard pooled pregnant human serum sample.

Effect of serum PAG on lymphocyte transformation and leucocyte migration

Sera obtained before and during stilboestrol treatment from four patients with prostatic carcinoma with no measurable PAG before treatment were used to determine their effects on (i) lymphocyte transformation induced by PHA and PPD and (ii) leucocyte migration inhibition.

(i) These were carried out on lymphocytes from a single normal individual known to be Mantoux-positive using PHA and PPD as transforming agents. The method used was

similar to that described previously (Yeung Laiwah, 1971). Transformation was carried out in pooled AB serum (West of Scotland Regional Blood Transfusion Service) as control, and compared with tests using sera obtained before and during stilboestrol treatment.

(ii) A modified version of the technique used by Bendixen & Søborg (1969) was employed. Peripheral leucocytes from a known strongly Mantoux-positive volunteer were incubated at 37°C for 1 hr in paired tubes containing normal serum or serum from patients with prostatic carcinoma. PPD was then added to one test tube of each pair to a final concentration of 100 μg protein/ml while a corresponding volume of Eagle's medium was added to the other tube. The cells were further incubated for 2 hr. They were subsequently put in capillary tubes, spun down and allowed to migrate in culture chambers containing Eagle's medium enriched with 10% foetal calf serum. Leucocyte migration was measured by planimetry under a projection microscope by an observer who did not know the details of the test samples. Results were expressed as a migration index (MI):

$$\text{MI} = \frac{\text{Mean migration of leucocytes incubated in patient's serum}}{\text{Mean migration of leucocytes incubated in normal serum.}}$$

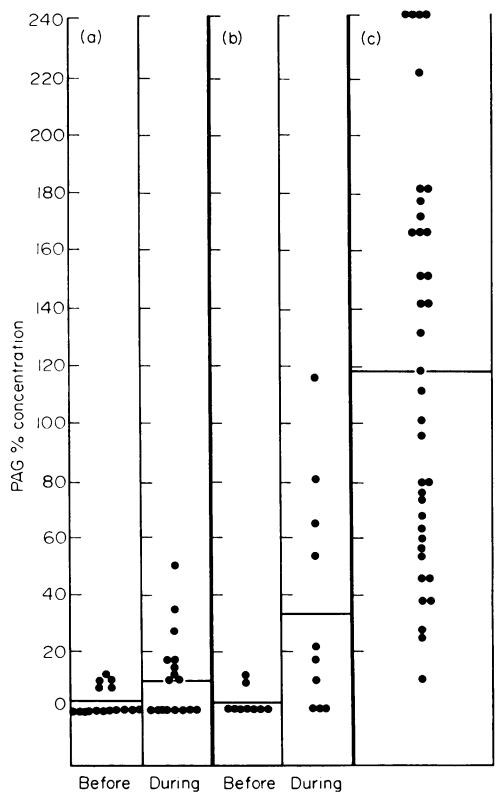

FIG. 1. Serum PAG levels in healthy females before and during administration of (a) combined oestrogen/progestogen oral contraceptives and in males before and during (b) stilboestrol therapy for prostatic carcinoma. The serum levels of PAG in (c) normal pregnancy are shown for comparison.

The mean migration of four to six capillary tubes was measured for each serum and each serum was examined in duplicate in each experiment.

The Student's *t*-test and χ^2 tests were used for statistical analysis where appropriate.

RESULTS

Serum PAG concentration

Individual serum PAG levels before and during administration of combined oestrogen/progestogen oral contraceptives are shown diagrammatically in Fig. 1 with the levels in

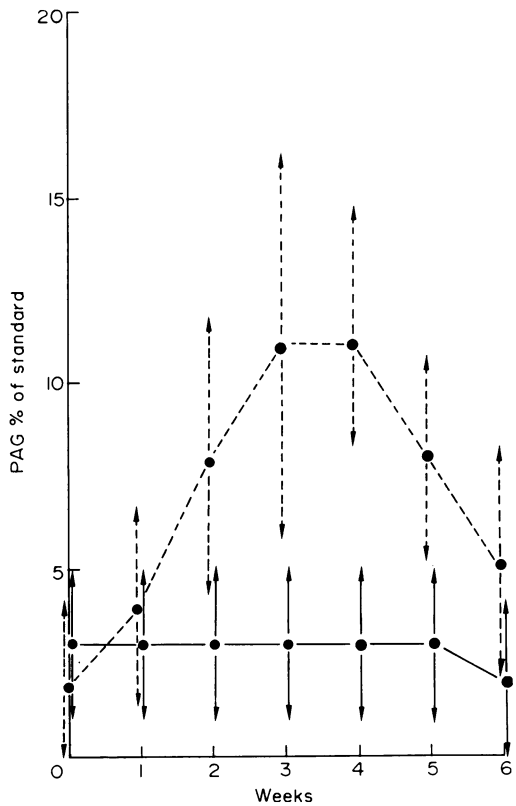

FIG. 2. Serum PAG levels in two groups of healthy females who received (- - -) oestrogen or (———) progestogen. Mean concentration (± SD) is shown. The serum levels are expressed as a percentage of a pooled pregnant human serum.

normal pregnancy (mean concentration 117·4%) shown for comparison. The mean concentration before treatment is 2·8% and during treatment 10·5% ($P<0·025$). However, only nine out of the eighteen females have measurable quantities of this globulin during treatment compared with five before treatment ($P<0·50$).

It is clear from Fig. 2 that administration of mestranol (oestrogen) leads to a marked increase in serum PAG concentration ($P<0·0005$), maximal after 3 or 4 weeks of therapy. Of the fourteen females taking this preparation only three had detectable levels of this

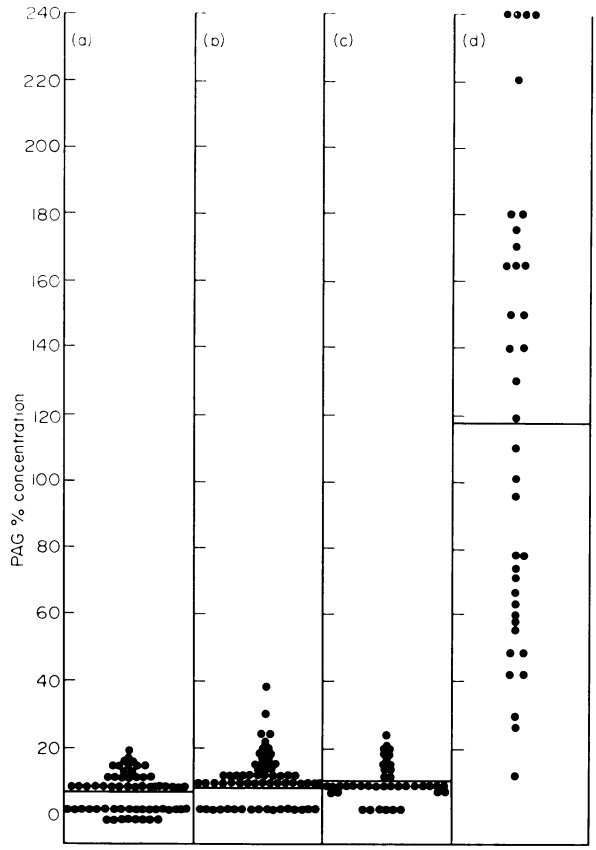

FIG. 3. Serum PAG levels in patients with (a) primary biliary cirrhosis, in (b) hospital in-patients (matched for age and sex with primary biliary cirrhosis patients) and in (c) patients with various malignant diseases. The serum levels of PAG in (d) normal pregnancy are shown for comparison.

TABLE 1. Effect of serum from patients with prostatic carcinoma, before and during stilboestrol therapy on lymphocytic responsiveness and leucocyte migration

Patient	Serum PAG (% concentration)		PHA response (% control)		PPD response (% control)		Migration Index	
	Before	During	Before	During	Before	During	Before	During
1	0	80	58	60	18	16	45	71
2	0	64	60	42	9	13	49	61
3	0	21	55	154	17	24	46	43
4	0	16	42	31	9	41	66	46

protein before therapy compared with twelve after therapy ($P<0{\cdot}01$). Those females who received ethynodiol diacetate (progestogen) do not show any increase in the numbers having detectable levels of this protein (four out of fourteen).

A highly significant increase in PAG concentration ($P<0{\cdot}01$) is observed in the stilboestrol-treated males during treatment (see Fig. 1), mean concentration before treatment being 2·0% and during treatment 35·9%. Of the ten patients two had detectable levels of PAG before treatment while during treatment a further five were found to have measurable PAG levels ($P<0{\cdot}10$).

In Fig. 3 serum PAG levels in primary biliary cirrhosis (mean 6·4%), hospital in-patients (mean 7·3%) and malignant disease (mean 9·4%) are shown. The serum PAG concentration in all these groups is considerably less than that found in late pregnancy. Measurable quantities of PAG were present in 63% of patients with primary biliary cirrhosis, 74% of the hospital in-patients and 87% of the malignant disease patients.

Lymphocyte transformation and migration inhibition studies

Of the twelve sera from patients with primary biliary cirrhosis which had previously been shown to have serum inhibitory factors to lymphocytic transformation induced by PHA and PPD, eleven contained measurable quantities of PAG. No correlation, however, was found between serum PAG levels and the degree of depression of lymphocytic transformation by PHA and PPD.

The results of detailed studies in four stilboestrol-treated patients are summarized in Table 1. With one exception, there is marked depression of both PHA and PPD responses by sera obtained both before and during treatment, but no significant difference is observed between the two groups of sera. In the migration test there is no significant difference between the mean migration index with sera obtained before and during stilboestrol treatment.

DISCUSSION

MacLaren *et al.* (1966) have shown that PAG is present in the serum of 18% of normal males and females and that the serum levels are inherited. They have also shown that of twenty-four females taking combined oestrogen/progestogen oral contraceptives all had demonstrable levels of PAG, the levels in these subjects being of a similar order of magnitude to that found in late pregnancy. In our study only nine out of the eighteen females receiving similar combined oestrogen/progestogen oral contraceptives have demonstrable PAG after therapy, and the serum levels are only of the order of 10% of that found in late pregnancy. It is possible that duration of treatment might explain such a difference.

Our results also show that only the oestrogen component of the oral contraceptive preparation is responsible for increases in serum PAG, and are in keeping with previous findings of increases in other serum proteins, including thyroxine-binding globulin, ceruloplasmin and corticosteroid-binding globulin (Musa, Doe & Seal, 1967) and α_2-macroglobulin and transferrin (Horne *et al.*, 1971). It is of some interest however that the males on stilboestrol therapy show a more striking increase in serum PAG levels.

Although MacLaren *et al.* (1966) have demonstrated that serum PAG levels are inherited, it is clear that oestrogen containing preparations can lead to the appearance of detectable amounts of this protein in the serum. It would therefore seem likely, as suggested by these

authors, that those normal subjects who do not have measurable PAG in their serum probably have trace amounts which cannot be detected by conventional techniques, the oestrogen merely increasing the production of PAG.

To our knowledge there are no studies of PAG levels in disease states. In all the disease groups we have studied so far, namely primary biliary cirrhosis, hospital in-patients with miscellaneous diseases and a variety of malignant diseases, there is a marked increase in the incidence of PAG compared with sera from normal males and females. It is of special interest that the highest percentage of PAG positive sera (87%) is found in malignant disease, although the mean concentration is only slightly above that of the other two groups. Increased serum α_2-globulins are known to occur in tumour bearing hosts (Ashikawa *et al.*, 1971) and raised serum PAG levels could at least in part explain such an increase. Although our series is small there is a suggestion that the presence of PAG in malignant disease is associated with disseminated rather than localized tumours.

It is now well established that serum from a variety of conditions including malignant disease (Trubowitz, Masek & Del Rosario, 1966; Gatti, Garrioch & Good, 1970; Whittaker, Rees & Clark, 1971), secondary syphilis (Levene *et al.*, 1969), multiple sclerosis (Burns *et al.*, 1971) and severe tuberculosis (Heilman & McFarland, 1966) can depress the PHA response of human lymphocytes. However, it is not clear whether one or more serum factors are involved. In preliminary experiments using four paired PAG negative and PAG positive sera from stilboestrol-treated males, we have been unable to demonstrate that sera containing measurable quantities of PAG have a depressive effect on the mitogenic response following PHA or PPD stimulation of lymphocytes. In addition, no correlation was observed between serum PAG level and the degree of depression of lymphocyte transformation of PHA or PPD in a small series of primary biliary cirrhosis patients. In the leucocyte migration test while there is no difference in the mean migration index with sera, before and during stilboestrol treatment, it is perhaps noteworthy that the two sera containing the largest amounts of PAG (Table 1) interfere with the inhibition of leucocyte migration. Further studies are in progress to clarify these observations.

ACKNOWLEDGMENTS

We thank Dr P. W. Howie, Dr R. J. Weir and Mr J. M. Anderson for supplying sera. This study was supported by research funds of Glasgow University and a grant from the Secretary of State for Scotland.

REFERENCES

ASHIKAWA, K., INOUE, K., SHIMIZU, T. & ISHIBASHI, Y. (1971) An increase of serum alpha-globulin in tumor-bearing hosts and its immunological significance. *Jap. J. exp. Med.* **41**, 339.

BENDIXEN, G. & SØBORG, M. (1969) A leukocyte migration technique for *in vitro* detection of cellular (delayed type) hypersensitivity in man. *Dan. med. Bull.* **16**, 1.

BURNS, C.P., ARMENTROUT, S.A., GROSS, S., VAN DEN NOORT, S. & STJERNHOLM, R.L. (1971) The effect of a lymphotoxic factor in the sera of patients with multiple sclerosis and acute leukemia on the metabolism of normal and leukemic lymphocytes. *J. Lab. clin. Med.* **78**, 508.

COOPERBAND, S.R., BONDEVIK, H., SCHMID, K. & MANNICK, J.A. (1968) Transformation of human lymphocytes: Inhibition by homologous alpha-globulin. *Science*, **159**, 1243.

FAHEY, J.L. & MCKELVEY, E.M. (1965) Quantitative determination of serum immunoglobulins in antibody-agar plates. *J. Immunol.* **94**, 84.

FIELD, E.J. & CASPARY, E.A. (1971) Lymphocytic reactivity in cancer. *Lancet*, **ii**, 877.

GATTI, R.A., GARRIOCH, D.B. & GOOD, R.A. (1970) Depressed PHA responses in patients with non-lymphoid malignancies. *Proceedings of Fifth Leukocyte Conference* (Ed. by J. E. Harris), pp. 339–357. Academic Press, New York.

GOUDIE, R.B., MACSWEEN, R.N.M. & GOLDBERG, D.M. (1966) Serological and histological diagnosis of primary biliary cirrhosis. *J. clin. Path.* **19**, 527.

HEILMAN, D.H. & MCFARLAND, W. (1966) Inhibition of tuberculin-induced mitogenesis in cultures of lymphocytes from tuberculous donors. *Int. Arch. Allergy*, **30**, 58.

HORNE, C.H.W., HOWIE, P.W. & GOUDIE, R.B. (1970) Serum alpha$_2$-macroglobulin, transferrin, albumin and IgG levels in pre-eclampsia. *J. clin. Path.* **23**, 514.

HORNE, C.H.W., MALLINSON, A.C., FERGUSON, J. & GOUDIE, R.B. (1971) Effects of oestrogen and progestogen on serum levels of α_2-macroglobulin, transferrin, albumin and IgG. *J. clin. Path.* **24**, 464.

MACSWEEN, R.N.M., HORNE, C.H.W., MOFFAT, A.J. & HUGHES, H. (1972) Serum protein levels in primary biliary cirrhosis. *J. clin. Path.* **25**, 789.

KASAKURA, S. (1971). A factor in maternal plasma during pregnancy that suppresses the reactivity of mixed leukocyte cultures. *J. Immunol.* **107**, 1296.

LEVENE, G.M., TURK, J.L., WRIGHT, D.J.M. & GRIMBLE, A.G.S. (1969) Reduced lymphocyte transformation due to a plasma factor in patients with active syphilis. *Lancet*, **ii**, 246.

MACLAREN, J.A., REID, D.E., KONUGRES, A.A. & ALLEN, F.H. JR. (1966) Pa 1, a new inherited alpha-2-globulin of human serum. *Vox Sang.* **11**, 553.

MANCINI, G., CARBONARA, A.O. & HEREMANS, J.F. (1965) Immunochemical quantitation of antigens by single radial immunodiffusion. *Immunochemistry*, **2**, 235.

MANNICK, J.A. & SCHMID, K. (1967) Prolongation of allograft survival by an alpha globulin isolated from normal blood. *Transplantation*, **5**, 1231.

MUSA, B.U., DOE, R.P. & SEAL, U.S. (1967) Serum protein alterations produced in women by synthetic estrogens. *J. clin. Endocr. Metab.* **27**, 1463.

SCHEUER, P.J. (1967). Primary biliary cirrhosis. *Proc. roy. Soc. Med.* **60**, 1257.

SHERLOCK, S. (1968) In: *Diseases of the Liver and Biliary System.* 4th ed. (rev. 3rd ptg, 1971), Blackwell Scientific Publications, Oxford.

SMITH, J.K., CASPARY, E.A. & FIELD, E.J. (1972) Immune responses in pregnancy. *Lancet*, **i**, 96.

STIMSON, W. (1972) Transplantation—Nature's success. *Lancet*, **i**, 684.

THOMPSON, A., HORNE, C.H.W., STEELE, H. & GOUDIE, R.B. (1969) Concentrations of serum α_2-macroglobulin and twins. *Nature* (*Lond.*), **221**, 289.

TRUBOWITZ, S., MASEK, B. & DEL ROSARIO, A. (1966) Lymphocyte response to phytohaemagglutinin in Hodgkin's disease, lymphocytic leukaemia and lymphosarcoma. *Cancer* (*Philad.*), **19**, 2019.

WHITTAKER, M.G., REES, K. & CLARK, C.G. (1971) Reduced lymphocyte transformation in breast cancer. *Lancet*, **i**, 892.

YEUNG LAIWAH, A.A.C. (1971) Lymphocyte transformation by Australia antigen. *Lancet*, **ii**, 470.