Immunology, 1972, **23**, 53.

Restrictions Among Heavy and Light Chain Determinants of Granulocyte-Specific Antinuclear Factors

A. Wiik and E. Munthe

Immunological Laboratory, University Clinic of Infectious Diseases, Blegdamshospitalet, Copenhagen, Denmark and
Research Institute of Immunology and Rheumatology, Rikshospitalet, Oslo, Norway

(*Received* 25*th August* 1971)

Summary. In fifty-five rheumatoid arthritis sera with positive granulocyte-specific antinuclear factors (GS-ANF) tests were made to further characterize these antibodies. All sera contained GS-ANF of the IgG class and most of the sera also of the IgM and IgA classes, whereas only about 10 per cent of the sera contained GS-ANF of the IgD class. Most of the sera contained GS-ANF carrying both κ and λ light chain determinants, but in five cases only one of the light chain subclasses could be found.

The distribution of the GS-ANF among the four subclasses of IgG showed marked variations. From one to three subclasses could be lacking or noticeably depressed. There was no predominance of any one or two subclasses. Complement (C_3) fixing properties correlated with GS-ANF of the IgG_1 and/or IgG_3 subclasses. These properties make the GS-ANF interesting as possible pathogenic factors in rheumatoid arthritis.

Some evidence is presented that the GS-ANF may be directed against several different antigens in the polymorphonuclear granulocyte nuclei.

INTRODUCTION

Granulocyte-specific antinuclear factors (GS-ANF) are antibodies which are able to combine with the nuclei of mature polymorphonuclear granulocytes, monocytes and eosinophils *in vitro* in the indirect immunofluorescence test. GS-ANF were originally recognized as a nosological entity by Faber, Elling, Norup, Mansa and Nissen in 1964, when they described the occurrence of GS-ANF in the serum of a patient exhibiting the characteristic features of Felty's syndrome (Faber *et al.*, 1964). Later on the GS-ANF have been found in all sera of patients with Felty's syndrome tested by the same technique, in about 75 per cent of the sera of patients with rheumatoid arthritis and in about one third of the sera of patients with systemic lupus erythematosus, often hidden by simultaneously occurring organ-nonspecific ANF (ON-ANF) (Faber and Elling, 1966; Elling, 1966; Elling, Graudal and Faber, 1967). GS-ANF has been found in IgG, IgA and the IgM classes (Elling and Graudal, 1968; Wiik and Faber, 1969) and usually carries both κ and λ chains (Elling, 1970; Wiik and Munthe, 1971). The antibodies react with nuclear antigens through

Correspondence: Dr Allan Wiik, Immunological Laboratory, University Clinic of Infectious Diseases, Blegdamshospitalet, Blegdamsvej 3, 2200 Copenhagen N, Denmark.

the Fab part of the molecules (Wiik and Munthe, unpublished data). Few GS-ANF possess complement C_3 fixing properties in the indirect immunofluorescence test (Wiik and Munthe, 1971).

The reactivity of the GS-ANF is specific in that the antibodies react with the nuclei of mature polymorphonuclear granulocytes, large mononuclear cells resembling monocytes and eosinophils, but considerably less or not at all with the precursors of these cells. The antibodies do not react with lymphocytes or other cells of human or animal origin (Elling, Graudal and Faber, 1967; Wiik and Faber, 1969). The specificity has been ascertained by cross absorption experiments using isolated nuclei from granulocytes, lymphocytes and human thyroid cells for the absorption of sera containing GS-ANF, ON-ANF or both GS-ANF and ON-ANF (Elling, 1966). The nuclear antigens involved in the reaction are unknown. Treatment of the leucocytes with DNase indicates that both DNase-labile and DNase-stabile antigens are involved (Wiik and Faber, 1969) and we have found that they are easily eluted with isotonic salt solution (Wiik, unpublished data). Several morphologically different fluorescence staining patterns have been recognized probably representing reactions between different nuclear antigens and the corresponding antibodies (Wiik and Faber, 1969).

The present investigation was undertaken to further characterize the heterogeneity of GS-ANF. We studied the distribution of GS-ANF in the four main immunoglobulin classes, the four subclasses of IgG and the two subclasses of light chains. Furthermore we looked for a possible relationship between the IgG subclass distribution, serum antiglobulin activity and complement C_3 fixing properties of GS-ANF. The introduction of a refined leucocyte preparation technique was another reason for re-evaluating some of the data on GS-ANF.

The results have been presented recently in abstract form (Wiik and Munthe, 1971).

MATERIALS AND METHODS

Human sera

Fifty-five sera containing GS-ANF were derived from fifty-four patients most of whom had definite or classical rheumatoid arthritis according to the ARA criteria (Committee of American Rheumatism Association, 1959). Previous investigations had shown that these sera contained only GS-ANF. Several sera from normal blood donors served as negative controls. All sera were stored at $-20°$ until tested.

Rabbit antisera

Specific anti-γ chain serum was obtained by absorbing antisera raised against human IgG with excess amounts of pepsin digested IgG. Specific anti-μ, anti-α and anti-δ chain sera were purchased from Nordic Pharmaceuticals and Diagnostics, Tilburg. Anti-IgG subclass sera were made by immunization of rabbits with isolated IgG myeloma proteins and were absorbed with excess amounts of IgG lacking the respective subclass antigen. Anti-C_3 serum was produced as described elsewhere (Munthe and Natvig, 1971b). A commercial antiserum against C_3 was also used (Nordic).

Fluorochrome labelling

IgG isolated from antiserum by gel filtration on DEAE-Sephadex or DEAE-cellulose columns was labelled with fluorescein isothiocyanate (Baltimore Biological Laboratories).

Labelling, removal of free dye and absorption with calf liver acetone powder or gel filtration through DEAE cellulose was performed according to standard procedures (Nairn, 1969; Hijmans, Schuit and Klein, 1969). The specificity of the conjugates was tested on monoclonal bone marrow plasma cells and by haemagglutination and/or immunodiffusion as described earlier (Munthe and Natvig, 1971a; Munthe and Natvig, 1971b). The commercial antisera were furthermore tested by immunoelectrophoresis. Most of the anti-subclass conjugates were applied at 4–8 units concentration to the respective myeloma plasma cells.

Staining procedure

Indirect immunofluorescence technique was employed using normal peripheral blood leucocytes as nuclear substrate. Leucocytes were isolated from defibrinated blood by the use of a dextran-isopaque gradient at 1 ***g*** according to the method described by Bøyum (1964). The floating cells in the serum layer on top of the gradient containing 5000–7000 leucocytes/μl were washed in phosphate buffered saline (PBS), pH 7·4 and the cells were smeared on glass slides, carefully defatted by washing in absolute alcohol. The cells were fixed on the slides at +4° for 5 minutes in absolute alcohol. After drying the slides were incubated with the human sera in a moist chamber at room temperature for 30 minutes, followed by cautious washing and immersion in PBS twice for ten minutes. Specific conjugates were applied and the slides were again incubated for thirty minutes followed by a second set of washings and immersions. Cover glasses were finally mounted with a mixture of glycerol and PBS (1:1) and the slides were examined within a few hours. The anti-complement test was performed according to the technique of Goldwasser and Shepard (1958).

Microscopy and microphotography

A Leitz Orthoplan microscope mounted with equipment for incident light illumination according to Ploem was used (Ploem, 1967). Excitation light source was a HBO 200 mercury lamp. Primary filters were a 1·5-mm BG 12 and a 3-mm BG 38 glass filter, an AL 470-nm or KP 490-nm interference filter. A TK 495-nm dicroic mirror was used. Secondary filters were a 1-mm 510 or 530-nm glass filter. Microphotographs were taken as described earlier (Munthe and Natvig, 1971a) using Anscochrome 500 colour film.

DNase-treatment of leucocytes

DNase I from bovine pancreas (Worthington Corporation, Freehold, New Jersey) 1 mg/ml in PBS was applied after fixation of the cells and coupling of GS-ANF to the nuclei. The slides were incubated in a moist chamber at +37° for 1 hour. After this treatment no Feulgen positive material could be detected in the nuclei by light microscopy or Feulgen fluorescence microscopy (Böhm and Sprenger, 1968). Control treatment was performed with PBS without enzyme.

Haemagglutination tests

Rheumatoid factors were detected with red cells sensitized with anti-Rh Ripley and by a modified Waaler-Rose test. Antibodies to the pepsin site of IgG (pepsin agglutinators) were detected with red cells coated with pepsin digested anti-Rh Ripley antibodies as previously described (Munthe, 1970).

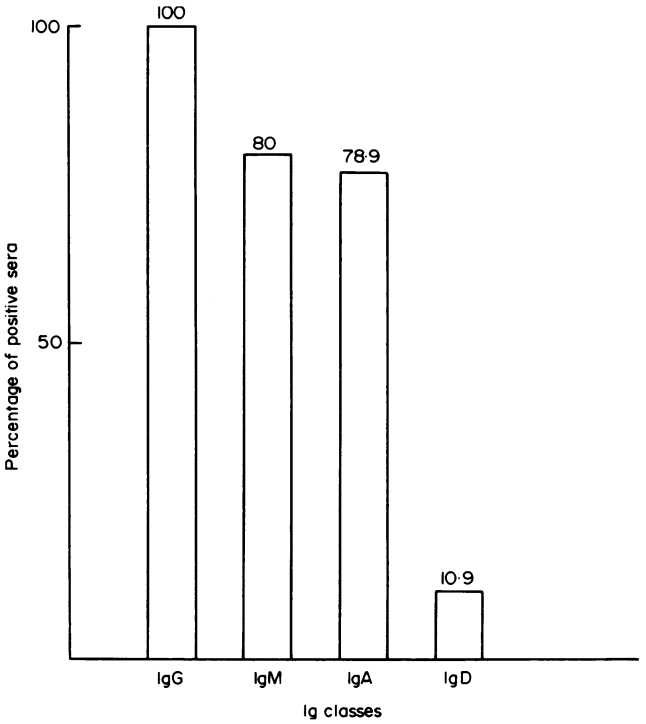

FIG. 1. Occurrence in GS-ANF in the four main Ig Classes.

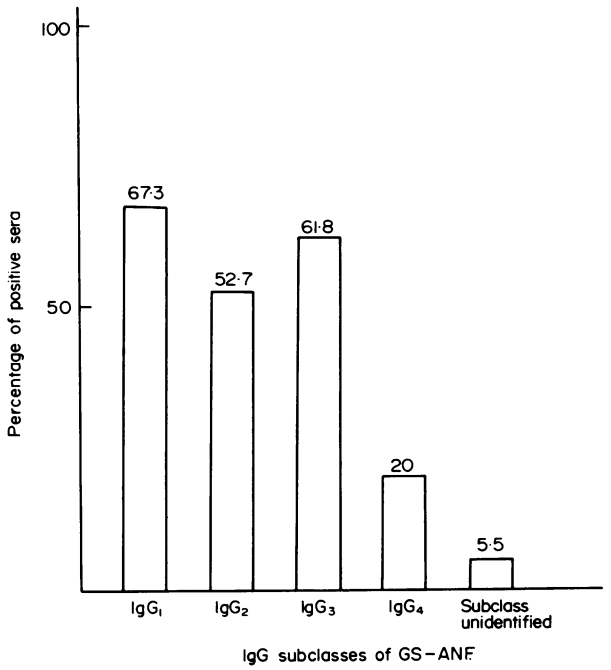

FIG. 2. Occurrence of GS-ANF in the four subclasses of IgG.

RESULTS

Fig. 1 depicts the occurrence of GS-ANF in the four main immunoglobulin classes expressed as percentages of positive sera and shows that nearly all the sera contained GS-ANF of more than one immunoglobulin class, predominantly IgG, IgM and IgA.

The percentage of sera containing GS-ANF in the respective subclasses of IgG is shown in Fig. 2. Antibodies were most frequent in subclasses 1, 2 and 3. In three sera the subclass

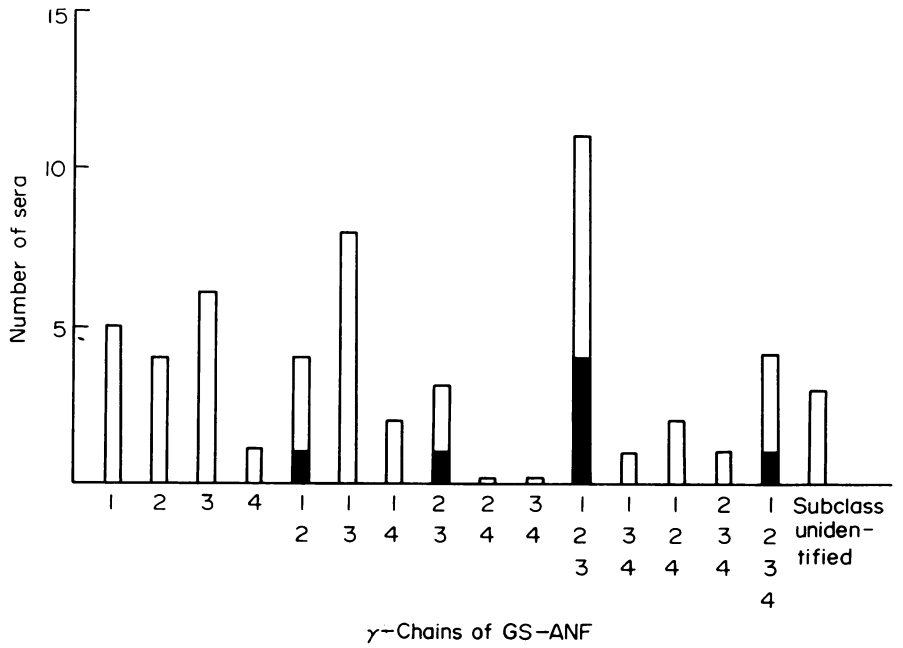

FIG. 3. IgG H-chain subclass combinations of GS-ANF in relation to complement C_3-fixing properties. Open bar, negative C_3-fixation; Solid bar, positive C_3-fixation.

could not be identified. The subclass distribution of IgG GS-ANF in the sera is shown in Fig. 3, which also shows the results of the immunofluorescent test for complement fixation. Only sera containing antibodies of IgG_1 and/or IgG_3 subclasses had complement fixing properties. No correlation was found between subclass composition and presence of anti-immunoglobulins detected by the Ripley coat agglutination test, the modified Waaler-Rose test or the pepsin agglutinator test.

Table 1 shows the light chain determinants detected in the GS-ANF in fifty sera. In five sera only one light chain subclass could be demonstrated despite four repeated examinations. Table 2 shows that the heavy chain class and subclass determinants of the GS-ANF in these sera were heterogeneous.

TABLE 1

LIGHT CHAIN SUBCLASSES OF GS-ANF

Both κ and λ	Only κ	Only λ	Undetermined	Investigated sera
44 (88 per cent)	2 (4 per cent)	3 (6 per cent)	1 (2 per cent)	50 (100 per cent)

TABLE 2

HEAVY CHAIN CLASS AND SUBCLASS DETERMINANTS OF GS-ANF CARRYING ONLY ONE DETECTABLE LIGHT CHAIN SUBCLASS DETERMINANT

Serum No.	L-chain.	H-chain
45 802	κ	γ§, μ, α,.
48 807	κ	γ_2, γ_3, γ_4, μ, α.
53 733	λ	γ_2, μ, α.
43 294	λ	γ_2, γ_3, α, δ.
49 459	λ	γ_1, γ_3, μ, α.

§ H-chain subclass could not be identified.

Some sera produced similar nuclear staining patterns with all the conjugates used, others gave different patterns with different conjugates. This was true for both class and subclass specific conjugates. In order to elucidate whether the different staining patterns might express antibodies to different antigens in the granulocyte nuclei, six sera containing GS-ANF of all four subclasses were tested on both DNase-treated and control-treated leucocytes. The negative reaction of four sera tested on DNase-treated leucocytes is shown in Table 3.

TABLE 3

REACTIVITY OF SIX GS-ANF SERA WITH DNASE-TREATED AND UNTREATED LEUCOCYTES

	Subclass of GS-ANF	Serum I	II	III	VIII	IX	X
Untreated* leucocytes	IgG_1	+	+	+	+	+	+
	IgG_2	+	+	+	+	+	+
	IgG_3	+	+	+	+	+	+
	IgG_4	+	+	+	+	+	+
DNase-treated leucocytes	IgG_1	+	+	+	+	+	+
	IgG_2	+	+	+	+	+	+
	IgG_3	+	+	+	+	+	+
	IgG_4	−	+	−	−	−	+

* Treated with PBS without DNase.

DISCUSSION

In this study the heterogeneity of GS-ANF was demonstrated, the antibodies occurring in the four main immunoglobulin classes, the four subclasses of IgG and the two subclasses of light chains. The frequent finding of GS-ANF in the IgG, IgM and IgA classes, in contrast to earlier results on 'granulocyte-reactive' ANF comprising both GS-ANF and ON-ANF (Elling and Graudal, 1968) may be due to several circumstances. First of all the sera used for this study reacted only with nuclei of polymorphonuclear granulocytes, monocytes and eosinophils. The technique used for the preparation of the nuclear substrate was different from the previous technique (Elling, 1966), in which repeated freezing and thawing of peripheral blood smears was used causing disruption of some of the cells. The use of absolute alcohol as a fixing and membrane denaturating agent may reveal previously

undetected antigens by rendering them more accessible to antibody, or conversely hide some antigens made unreactive by denaturation. The defibrination procedure proved to be favourable, possibly due to the mechanical effect on the leucocyte membranes. Removal of immunoglobulin from the leucocyte donor serum by washing the cells, and the use of leucocyte concentrates are obvious advantages. Differential counts of peripheral blood smear preparations compared with leucocyte concentrate smears are identical. In addition, the microscopical equipment and the conjugates have improved during the last few years.

The finding of GS-ANF in the IgD class and in the four subclasses of IgG has not been reported earlier. ON-ANF of the IgD class was found in a rather high proportion of sera from patients with systemic lupus erythematosus, namely 56 per cent (Ritchie, 1968) and 45 per cent (Watson, Heiner, Rose and Bootello, 1969). In systemic sclerosis and rheumatoid arthritis IgD ON-ANF were found in about 20 per cent of the sera (Ritchie, 1968). Very recently Kacaki, Budimir, Nikolić, Marinković and Berović (1971) reported finding IgD ON-ANF in 15 per cent of 100 ANF positive sera.

ON-ANF in all four subclasses of IgG has been described in sera from patients with systemic lupus erythematosus (Tojo, Friou and Spiegelberg, 1970) and the distribution of the anti-DNA nucleoprotein antibodies among the subclasses was estimated to be proportional to the total amounts of the individual IgG subclasses in normal sera. Our investigation is only qualitative, but the results show that there is no predominance of any subclass as described for antibodies to dextran (Yount, Dorner, Kunkel and Kabat, 1968) or anti-Rhesus antibodies (Natvig and Kunkel, 1967). Complement fixing properties are ascribed to antibodies of the subclasses IgG_1 and IgG_3 and to a lesser degree IgG_2 whereas IgG_4 does not fix complement (Müller-Eberhard, Hadding and Calcott, 1967). GS-ANF of the IgG_1, IgG_2 and IgG_3 subclasses were found in about the same number of sera whereas only one fifth of the sera contained antibodies of the IgG_4 subclass. The subclass distribution of the GS-ANF therefore does not account for the rarity of complement fixing properties. However in the few sera exhibiting complement fixation, a correlation was found with the presence of IgG_1 and/or IgG_3 antibodies.

In seventeen sera IgG GS-ANF were found in one subclass only. In five sera the GS-ANF apparently carried only one light chain subclass. Whether this is a true expression of selection of single subclasses is uncertain. As Barnett, Bakemeier, Leddy and Vaughan (1965) found, concentration of the immunoglobulins of the sera might reveal both κ and λ light chain determinants to be present. The same explanation may apply for our inability to show more than one heavy chain subclass of the GS-ANF, but unfortunately our supply of serum was too small for concentration experiments.

The finding of GS-ANF in so many immunoglobulin classes and subclasses of some sera could mean that not only nucleo-specific antibodies are recognized as ANF in the indirect immunofluorescence technique. As pointed out by Barnett, Condemi, Leddy and Vaughan (1964) anti-immunoglobulins bound to the nuclear antigen–antibody complex would be recognized as ANF. Absorption of rheumatoid factors by F II-coated latex particles caused a significant decrease of IgM ON-ANF in one out of seven of their sera. Our experiments with GS-ANF indicate a very close relationship between GS-ANF and rheumatoid factors capable of reacting with isologous aggregated IgG, perhaps in the form of circulating complexes (Wiik and Munthe, in preparation). Which of the immunoglobulins in the ANF test represent GS-ANF and which rheumatoid factors is thus still uncertain.

Previous and present studies show that GS-ANF is a heterogeneous population of antibodies directed against several different nuclear antigens. At least some of these antigens

can be found in monocytes, and some in eosinophils (Wiik and Faber, 1969) and in granulocytes of different animal species (Elling, 1970). The antigens are as yet unknown.

ACKNOWLEDGMENTS

We are grateful to Dr Jacob B. Natvig and Dr Viggo Faber for valuable advice and helpful criticism and for providing technical facilities.

We also thank Dr Kaj Kalbak and the Danish State Serum Institute for providing the sera, and Miss Helle Jareg and Mrs Tove Dietz for excellent technical assistance.

This study was supported in part by grants from the National Danish Association Against Rheumatic Diseases, the Norwegian Rheumatism Council and the Norwegian Women's Public Health Association.

REFERENCES

BARNETT, E. V., BAKEMEIER, R. F., LEDDY, J. P. and VAUGHAN, J. H. (1965). 'Heterogeneity of antinuclear factors in lupus erythematosus and rheumatoid arthritis.' *Proc. Soc. exp. Biol. (N.Y.)*, **111**, 803.

BARNETT, E. V., CONDEMI, J. J., LEDDY, J. P. and VAUGHAN, J. H. (1964). 'Gamma$_2$, gamma$_{1A}$, and gamma$_{1M}$ antinuclear factors in human sera.' *J. clin. Invest.*, **43**, 1104.

BÖHM, N. and SPRENGER, E. (1968). 'Fluorescence Cytophotometry: A valuable method for the quantitative determination of nuclear Feulgen-DNA.' *Histochemie* **16**, 100.

BØYUM, A. (1964). 'Separation of white blood cells.' *Nature (Lond.)*, **204**, 793.

Committee of American Rheumatism Association on Criteria for rheumatoid arthritis (1959). 'Primer on the rheumatic diseases.' *J.A.M.A.*, **171**, 1213.

ELLING, P. (1966). 'Reaction of antinuclear factors with polymorphonuclear leucocytes.' *Acta path. microbiol. scand.* **68**, 281.

ELLING, P. (1970). *Antinuclear antibodies.* Thesis, Copenhagen.

ELLING, P. and GRAUDAL, H. (1968). 'IgG, IgA and IgM granulocyte-reactive antinuclear factors in rheumatoid arthritis.' *Acta med. scand.* **183**, 25.

ELLING, P., GRAUDAL, H. and FABER, V. (1967). 'Organ-specific and organ-nonspecific autoantibodies in rheumatoid arthritis.' *Acta med. scand.* **182**, 707.

FABER, V. and ELLING, P. (1966). Leucocyte-specific anti-nuclear factors in patients with Felty's syndrome, rheumatoid arthritis, systemic lupus erythematosus and other diseases.' *Acta med. scand.* **179**, 257.

FABER, V., ELLING, P., NORUP, G., MANSA, B. and NISSEN, N. I. (1964). 'An antinuclear factor specific for leucocytes.' *Lancet*, **ii**, 344.

GOLDWASSER, R. A. and SHEPARD, C. C. (1958). 'Staining of complement and modification of fluorescent antibody procedures.' *J. Immunol.* **80**, 122.

HIJMANS, W., SCHUIT, H. R. E. and KLEIN, F. (1969). 'An immunofluorescence procedure for the detection of intracellular immunoglobulins.' *Clin. exp. Immunol.* **4**, 457.

KAGAKI, J., BUDIMIR, M., NIKOLIĆ, J., MARINKOVIĆ, D. and BEROVIĆ, Z. (1971). 'Immunoglobulin D in some rheumatic diseases.' *VII European Rheumatology Congress, Brighton* 1971. Abstract 37–8.

MUNTHE, E. (1970). 'Relationship between IgG complexes and anti-IgG antibodies in rheumatoid arthritis.' *Acta Rheum. Scand.* **16**, 240.

MUNTHE, E. and NATVIG, J. B. (1971a). 'Characterization of IgG complexes in eluates from rheumatoid tissue.' *Clin. exp. Immunol.* **8**, 249.

MUNTHE, E. and NATVIG, J. B. (1971b). 'Detection of genetic markers of human immunoglobulins by immunofluorescence technique.' *Ann. N.Y. Acad. Sci.* **177**, 326.

MÜLLER-EBERHARD, H. J., HADDING, U. and CALCOTT, M. A. (1967). 'Current problems in complement research.' *Immunopathology Mechanisms of Inflammation Induced by Immune Reactions*, p. 179. Grune & Stratton, New York.

NAIRN, R. C. (1969). 'Fluorescent Protein Tracing' 3rd ed. (Ed. by R. C. Nairn) p. 22, Livingstone, Edinburgh, & London.

NATVIG, J. B. and KUNKEL, H. G. (1967). 'Detection of genetic antigens utilizing gamma globulins coupled to red blood cells.' *Nature (Lond.)*, **215**, 68.

PLOEM, J. S. (1967). 'The use of a vertical illuminator with interchangeable dichroic mirrors for fluorescence microscopy with incident light.' *Z. wiss. Mikrosk.* **68**, 129.

RITCHIE, R. F. (1968). 'IgD antinuclear antibodies.' *Arthr. and Rheum.* **11**, 506.

TOJO, T., FRIOU, G. J. and SPIEGELBERG, H. L. (1970). 'Immunoglobulin G subclass of human antinuclear antibodies.' *Clin. exp. Immunol.*, **6**, 145.

WATSON, I., HEINER, D., ROSE, B. and BOOTELLO, A. (1969). 'The demonstration of IgD antinuclear antibody activity in systemic lupus erythematosus.' *Clin. Res.* **17**, 362.

WIIK, A. and FABER, V. (1969). 'Multiple leukocyte-specific antinuclear antibodies in rheumatoid arthritis.' *XII Congr. Rheumat. Internat., Prague* 1969. Abstract 632.

WIIK, A. and MUNTHE, E. (1971). 'Selectivity between IgG subclasses in granulocyte-specific antinuclear factors of rheumatoid arthritis sera.' *VII European Rheumatology Congress, Brighton* 1971. Abstract 17–3.

YOUNT, W. J., DORNER, M. M., KUNKEL, H. G. and KABAT, E. A. (1968). 'Studies of human antibodies. VI. Selective variations in subgroup composition and genetic markers.' *J. exp. Med.*, **127**, 633.