HUMAN ANTINUCLEAR ANTIBODIES

An Immunological, Clinical and Histochemical Study

by

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FOREWORD

All the immunological investigations on antinuclear antibodies and most of the biochemical preparations described in this thesis were performed and recorded personally during the period 1960 - 1963, whilst I was M.R.C. Clinical Research Fellow in the Division of Immunology, National Institute for Medical Research, Mill Hill, London and Lecturer in Pathology at the University and Western Infirmary, Glasgow.

Much of this work has been presented at meetings of various scientific societies, namely:

(a) The Immunological Society of Great Britain

(b) The Pathological Society of Great Britain and Ireland

(c) The Royal College of Physicians and Surgeons, Glasgow:
Watson Lecture.

(d) The American Rheumatism Association

(e) The Tissue Culture Society

(f) The Antibody Club of London

(g) The National Institutes of Health, Bethesda, Maryland, U.S.A.:
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and/or published in scientific journals, namely:

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INTRODUCTION

This thesis describes investigations on antinuclear antibodies which are found most commonly in patients with "connective tissue diseases". This has been a laboratory study of disordered immunological reactions so that it would be inappropriate to give a long historical account of the clinical and morbid anatomical features of these diseases. These aspects of the subject have been reviewed by Klemperer et al. (1942), Harvey et al. (1954), Bloch and Bunim (1963), Mackay and Burnet (1963), Peterson and Good (1963), Rodnan (1963) and many other authors and it is now clear that there is considerable overlap of clinical and pathological abnormalities and of familial incidence between the various "connective tissue diseases".

The early tentative suggestions that there might be an abnormality of the immunological mechanism in these diseases were based on histological analogies to serum sickness (Rich and Gregory, 1947) or allergic disease (Teilum, 1948) or on clinical observations of systemic lupus erythematosus developing shortly after injection of foreign protein (Fox, 1943). The first direct observation of abnormal immunological reactivity in the "connective tissue diseases" was made in 1946 when Callender and Race demonstrated five separate anti-erythrocyte antibodies in a systemic lupus erythematosus patient who had had multiple blood transfusions; the tendency to produce multiple antibodies after transfusion (often to unusual blood groups)

has since been confirmed by Waller and Race (1951) and Kuhns and Bauerlein (1953). Rather surprisingly, this passed almost unnoticed and did not stimulate immunological investigation in the "connective tissue diseases".

The spark which set off the current interest in autoimmunity in these diseases was the demonstration by Hargraves et al. (1948) of the Lupus Erythematosus cell (L. E. cell) phenomenon which was subsequently shown to depend upon the presence of non-organ-specific antinuclear antibody in the patient's serum; this phenomenon will be discussed later.

Since the discovery of the L. E. cell phenomenon, non-organ-specific autoantibodies to other cellular antigens have been shown to be common in "connective tissue disease" patients by complement fixation techniques (Gajdusek, 1958; Mackay and Gajdusek, 1958; Asherson, 1959; Deicher et al., 1960; Hackett et al., 1960a; Hijmans et al., 1961; Pasnick et al., 1962), and by precipitin reactions (Jones, 1958; Anderson et al., 1961b and 1962).

In addition, tissue-specific antibodies have been detected less frequently in these diseases, anti-erythrocyte antibodies (Zoutendyk and Gear, 1951; Harvey et al., 1954; Cowling and Thomas, 1957; Sarles and Levin, 1959; Dacie, 1962), anti-leucocyte antibodies (Eyquem et al., 1955; Killman, 1957; Dausset et al., 1961; Engelfriet and van-Loghem, 1961), anti-platelet antibodies (Weinreich, 1957; Dausset et al., 1961), antibodies to clotting factors (Conley

and Hartmann, 1952; Margolius et al., 1961) and antithyroid antibodies (Anderson et al., 1961a; Hijmans et al., 1961) are now well recognised. Rheumatoid factor is commonest in rheumatoid arthritis but it is also present in a high proportion of patients with the other "connective tissue diseases" (Svartz and Schlossman, 1957; Ziff, 1957; Kellgren and Ball, 1959; Bloch and Bunim, 1963).

In many cases, the abnormal antibodies have been shown to react with the corresponding antigens in the patient's tissues so that they must be considered as true autoantibodies. In addition, however, the patients have an increased tendency to develop iso-antibodies (e.g. blood group antibodies, Callender and Race, 1946 or anti-RA precipitin, Anderson, 1963) but rather surprisingly these patients do not appear to over-react to exogenous antigens (Meiselas et al., 1961; Sarkany, 1961). It is thus obvious that in the "connective tissue diseases" there is a profound immunological disturbance with widespread loss of immunological tolerance. It is almost certain that some circulating antibodies are directly concerned in the pathogenesis of certain manifestations of the disease such as the depression of circulating blood cells by autoantibodies to antigens on their cell surfaces. The significance of the non-organ-specific autoantibodies is less clear since transplacental transfer of these antibodies does not appear to produce the disease in the infant (Chapter 9) and in any patient the presence and titre of these antibodies does not appear to be directly related to the severity of

of the disease (Chapter 8). There is, however, some evidence that complexes of non-organ-specific autoantibodies with the corresponding antigens may be important in the causation of the renal lesions of systemic lupus erythematosus but not in the other manifestations of the disease (Raitt and Holman, 1962).

If autoimmunity is responsible for the pathogenesis of the "connective tissue diseases" (and this is still undecided) then it is highly probable that the initiating mechanism will prove to be delayed hypersensitivity but this cannot be investigated in man with presently available methods. The circulating autoantibodies may prove to be merely epiphenomena but it is possible that they may react in lesions produced by delayed hypersensitivity to aggravate and prolong the tissue damage since fixed γ globulin and complement have been demonstrated in the lesions of the "connective tissue diseases" (Mellors et al., 1957; Vazquez and Dixon, 1957; Lachmann et al., 1962). Furthermore, the serum complement level is commonly lowered in these patients (Vaughan et al., 1951; Elliot and Mathieson, 1953; Ellis and Felix-Davis, 1959; Lange et al., 1960; Townes et al., 1963b) due mainly to a fall in the 2nd and 4th components (Morse et al., 1961).

The L. E. Cell Phenomenon

The L. E. cell was first recognised by Hargraves et al. in 1948 in concentrated preparations of bone marrow from patients with systemic lupus erythematosus. This cell is a polymorphonuclear

leucocyte which has been distorted by the phagocytosis of a rounded amorphous mass of nuclear material. The inclusion body stains rather redder than normal nuclei in Geimsa preparations and shows no evidence of a chromatin pattern. The morphological appearances have been studied recently with the electron microscope and it has been shown that the inclusion body is surrounded by a cytoplasmic membrane such as is seen around a phagocytosed particle (Maldonado et al., 1963).

It was soon realised that L. E. cells do not usually occur in the living patient but are produced during incubation of marrow (Haserick and Sundberg, 1948; Hargraves, 1949) or buffy coat of peripheral blood from systemic lupus erythematosus patients (Sundberg and Liek, 1949; Hamburger, 1950). Under specialised conditions L. E. cells may occur *in vivo*. These cells have been found in the circulating blood in one patient in the terminal stages of acute systemic lupus erythematosus (Chomet *et al.*, 1953) and in another patient in bone marrow fixed immediately after aspiration (Schleicher, 1953). In systemic lupus erythematosus patients, they appear in experimentally induced inflammatory lesions (Perillie *et al.*, 1960), in cantharides blisters (Watson *et al.*, 1951) and in the blood during circulatory stasis (Sickley *et al.*, 1955). L. E. cells are found occasionally in the lesions of systemic lupus erythematosus (Guelf, 1950; Klemperer, 1952; Wilson *et al.*, 1961). The L. E. cell can be induced passively by application of lupus

erythematosus serum to abraded areas on the skin of normal individuals (Rodbuck and Berman, 1950).

The formation of L. E. cells has been shown to depend upon a plasma factor, since lupus erythematosus plasma could induce L. E. cell formation when incubated with normal buffy coat or marrow (Hargraves, 1949; Haserick and Bortz, 1949; Moffat et al., 1950), but this factor is also present in serum (Barnes et al., 1950; Gonyea et al., 1950; Eppes and Ludovic, 1951; Lee, 1951) and in defibrinated plasma (Eppes and Ludovic, 1951; Sukata and Conley, 1951). Serum fractionation, first by electrophoresis and later by ion-exchange chromatography and ultracentrifugation has shown that the L. E. cell factor is a γ globulin with sedimentation coefficient 7.0S (Berman et al., 1950; Haserick et al., 1950; Fallet et al., 1959; Larson et al., 1959). The thermal stability of the L. E. cell factor is similar to that of normal γ globulin (Hargraves, 1949; Haserick, 1950; Lee et al., 1951). The antigenicity of the L. E. cell factor is now known to be identical to that of normal human γ globulin (Holman and Deicher, 1959) and the early suggestion that it might be antigenically distinct (Haserick and Lewis, 1950; Low and Zetterström, 1955) have proved erroneous.

In 1951, Beerman emphasised that the L. E. cell phenomenon is a three component system; the plasma factor reacts with nuclear material which is subsequently phagocytosed by living cells. The three components will be discussed separately.

(1) Nuclear Material

The early experiments indicated that the nuclear component was derived from damaged cells and so techniques for the detection of the L. E. cells were devised in which cells were damaged mechanically to allow easy access of the L. E. cell factor to the nuclei (Zimmer and Hargraves, 1952; Zinkham and Conley, 1956).

(a) Source of nuclei Normal human leucocytes have been used in most experiments (Hargraves, 1949; Haserick and Bortz, 1949; Moffatt et al., 1950; Moyer and Fisher, 1950; Rohn and Bond, 1952; Stich et al., 1952; Rifkind and Godman, 1957) but frozen white cell rich plasma (German and Huber, 1958; Lachmann, 1961a), chronic myeloid leukaemia cells (Fisher and Moyer, 1950; Moyer and Fisher, 1950) and normal marrow and leucocytes from several mammalian species (Berman et al., 1950; Haserick, 1950; Carrera et al., 1954) are equally suitable. Ross and Wells (1953) have claimed that pigeon marrow is unsuitable but this has not been confirmed. Fragments of purified nucleohistone (Holman and Deicher, 1959), reconstituted nucleoprotein and partially digested spermatozoal nuclei (Lachmann, 1961b) have been used successfully as substrate for passive L. E. cell tests. A unique serum has been investigated by Svec and Kaplan (1963) who found that this L. E. cell factor would react only with physically traumatised nuclei.

(b) Modifications in the nuclear material during the production of the L. E. body The morphological changes of swelling and loss of

chromatin pattern were described clearly by Hargraves et al., (1948) who also showed that the bodies were Feulgen positive and therefore deduced that they contained deoxyribonucleic acid (DNA). There has been an isolated report that they contain ribonucleoprotein (Dameshek and Bloom, 1950) but this was not confirmed. The L. E. bodies showed an increased uptake of methyl green; this was interpreted by Lee et al. (1951) as indicating depolymerisation of DNA and this group later suggested that the L. E. cell factor activated intracellular deoxyribonuclease (DNase). Careful histochemical studies have now shown that the increased methyl green uptake is caused by a twofold increase in protein in the L. E. body and that the DNA is not depolymerised (Godman and Deitch, 1957a and b).

(2) Phagocytic cells

(a) Cell types The most common phagocytic cell is the polymorphonuclear leucocyte (Hargraves et al., 1948; Moyer and Fisher, 1950) but occasionally other cells may take up the L. E. body, e.g. monocytes, eosinophil leucocytes, basophil leucocytes or even myeloid leukaemia metamyelocytes or stab cells (Moyer and Fisher, 1950; Lee et al., 1951; Rohn and Bond, 1952).

(b) Accessory factor Phagocytosis can only occur in the presence of a heat-labile factor which is present in all normal and diseased sera; Formijne and van Seeren (1958) and Lachmann (1961b) considered that this factor was complement but Aisenberg (1959) suggested that it was another unidentified heat-labile constituent of serum.

(c) Speed of development of L. E. cells These cells are usually absent from freshly drawn blood and do not develop if blood is stored at low temperature although they can be found 20 min. after rewarming (Suksta and Conley, 1951). Haserick (1950) has shown that L. E. cells appear 12 to 13 min. after incubation and Lee et al. (1951) claim that they start to appear in small numbers after 2 min. but that the maximum numbers are not seen until the blood has been incubated for 20 min.

(3) L. E. serum factor

The observation that the L. E. cell factor was a 7.0S globulin (see p.11) suggested that it might be an antibody to cell nuclei and this was supported by the demonstration that the factor could be absorbed from lupus erythematosus serum by isolated cell nuclei (Miescher and Fauconnet, 1954). Later experiments suggested that the nuclear antigen was nucleohistone (DNA-histone complex) (Holman and Kunkel, 1957; Hijmans and Schuit, 1958; Klein et al., 1959) but the situation was obscured by Seligmann and Robineaux (1958) when they demonstrated weak L. E. cell promoting activity in γ globulin disassociated from the precipitate produced by the interaction of lupus erythematosus serum and purified DNA and by Aisenberg (1959) who made fluorescent antibody studies on lupus erythematosus sera and assumed that his results would apply to the L. E. phenomenon. In a series of careful investigations, Holman and Deicher (1959) have shown convincingly that the antigen is nucleohistone:

(i) The L. E. cell factor was absorbed by nucleohistone but not by DNA nor histone separately.

(ii) The L. E. cell factor could be dissociated from complexes with nucleohistone only after treatment with DNase and proteolytic enzymes.

(iii) The L. E. cell factor did not dissociate histone from nucleohistone.

Lachmann (1961b) has extended this work and shown that artificial DNA-protein complexes and partially digested nucleoprotamine could also act as antigen for the L. E. cell factor. In retrospect, it seems highly probable that Seligmann's (1958) observations have resulted from contamination of his DNA preparations by small quantities of nucleohistone.

It is thus concluded that the L. E. cell phenomenon is a biological test for the recognition of anti-nucleohistone, but it has the great disadvantage that the physicochemical conditions must be fairly rigidly controlled for the second stage of the reaction in order that the living polymorphonuclear leucocytes may be capable of phagocytosis. Furthermore, the test is relatively insensitive, it cannot be quantitated readily and the reading is somewhat subjective.

Antinuclear Antibodies

It is hardly surprising that research workers have turned to conventional immunological methods for further investigation of antinuclear antibodies, because the techniques are more sensitive,

more easily quantitated, less subjective and require much less rigidly controlled physico-chemical conditions than the L. E. cell test. The use of purely in-vitro test systems has stimulated research and the reports published before the start of my investigations will be reviewed in this introduction. More recent publications will be discussed in the subsequent chapters.

In 1955, Miescher demonstrated the reaction of sera from patients with systemic lupus erythematosus with isolated cell nuclei by a Coombs consumption test and this was confirmed by Engelfriet et al. (1959); these tests showed that the active serum factor had the antigenicity of human γ globulin and it was deduced that it was probably an antinuclear antibody. The reaction of the systemic lupus erythematosus serum factor with isolated cell nuclei was demonstrated by complement fixation tests by Robbins et al. (1957), Hijmans and Schuit (1958), Seligmann (1958), Ashorson (1959) and Holman et al. (1959): this was further evidence that the reaction was immunological.

This work was extended by investigation of the reaction of the antinuclear sera with certain purified constituents of cell nuclei and various systemic lupus erythematosus sera have been shown by several conventional immunological techniques to react specifically with the different nuclear constituents studied.

(i) Anti-nucleohistone has been demonstrated by complement fixation reactions (Robbins et al., 1957; Hijmans and Schuit, 1958; Scalettar et al., 1960; Seligmann, 1960), by passive haemagglutination (Miescher

and Strässle, 1957; Goodman et al., 1958; Goodman and Bowser, 1959; Lee and Epstein, 1960), by latex agglutination (Christian et al., 1958; Fessel et al., 1959), by the fluorescent spot test (Friou, 1958a and b; Crawford et al., 1959) and by a radioactive tracer technique (Friou, 1958c).

(ii) Anti-DNA by precipitin tests (Seligmann, 1957; Seligmann and Robineaux, 1958; Deicher et al., 1959; Barbu et al., 1960), by complement fixation reactions (Ceppellini et al., 1957; Robbins et al., 1957; Seligmann and Milgrom, 1957; Hijmans and Schuit, 1958; Pearson et al., 1958), by passive haemagglutination (Miescher and Strässle, 1957; Jokinen and Mäkitalo, 1959), by bentonite flocculation tests (Bozicevich et al., 1960; Kayhoe et al., 1960) and by cutaneous anaphylaxis (Deicher et al., 1960);

(iii) Anti-histone by complement fixation reactions (Holman et al., 1959).

(iv) Antibody to a phosphate buffer extract of isolated cell nuclei by complement fixation reactions (Holman et al., 1959).

In these experiments the emphasis has been on demonstration of the specificity of the reaction with nuclear components studied but Seligmann and Robineaux (1958) have shown that the serum factor reacting with DNA has the electrophoretic mobility of a γ globulin and Goodman et al. (1960) have shown that anti-nucleohistone is a 7.0S globulin in some sera and a macroglobulin in others. The evidence is therefore strongly in favour of accepting these serum

factors as antibodies. As no attempt had been made to demonstrate the reaction of these serum factors with antigen prepared from the patient's tissue, there was not sufficient evidence to prove that they were true autoantibodies, although this was highly probable from previous experience with the L. E. cell factor.

The fluorescent antibody method allows cytological localisation of the site of action of an antibody with a tissue antigen (Chapter 1). This technique had been used extensively to demonstrate the reaction of antinuclear antibodies with cell nuclei before mid 1960 (Friou, 1957; Holborow et al., 1957; Holman and Kunkel, 1957; Mellors et al., 1957; Alexander and Duthie, 1958; Bardawil et al., 1958; Friou, 1958a; Friou et al., 1958; Aisenberg, 1959; Calabresi et al., 1959; Holborow and Weir, 1959; Alexander et al., 1960; Baugh et al., 1960; Goodman et al., 1960). Throughout this work it had been assumed tacitly that all the antibodies had been reacting with the same nuclear antigen, despite the evidence from other investigators that multiple systems of antinuclear antibodies were involved. The investigations were, however, very useful as they demonstrated that the antinuclear antibodies were true/auto-antibodies (Alexander and Duthie, 1958) and that nucleohistone was an important nuclear antigen (Friou, 1958a; Holborow and Weir, 1959).

This thesis records my investigations on human antinuclear antibodies using the fluorescent antibody technique. This work can be subdivided into four parts thus:-

Part I Development of a simple quantitative technique for distinguishing four systems of antinuclear antibodies in a fluorescent antibody test.

Part II Immunological observations on nature of the nuclear auto-antigens and the physico-chemical properties of the antinuclear antibodies.

Part III Clinical studies -

(a) incidence of antinuclear antibodies in random hospital patients not known to be suffering from autoimmune disease.

(b) incidence of these antibodies in patients with "connective tissue" and certain other diseases.

(c) observations on the effect of transplacental transfer of the antibody from mothers with "connective tissue disease" to their normal infants.

Part IV Experiments in which selected human sera containing characterised antinuclear antibodies were used as highly specific histochemical reagents.

PART 1

CHAPTER 1

MORPHOLOGICAL PATTERNS OF AUTOIMMUNE NUCLEAR FLUORESCENCE

In 1934, Marrack showed that an antibody protein could be combined chemically with an ordinary dye without complete loss of its capacity to react specifically with its antigen. This was confirmed by Coons et al. (1941), who attempted to use dye-conjugated-antibody to trace the corresponding antigen in biological material. The ordinary dye used by Marrack was too insensitive, but fluorescent dyes could be detected in very much lower concentration and proved suitable for localisation of the small amount of antibody attached to the antigen in tissue. In subsequent experiments, Coons et al. (1942) introduced fluorescein as a fluorescent label. This dye has since been used extensively as it gives bright yellow-green fluorescence which is readily distinguishable from tissue auto-fluorescence.

The antibody retains its specific immunological properties when conjugated with a fluorescent dye and it becomes a sensitive reagent for the detection of its antigen. It must be remembered that the specificity of a fluorescent antibody is immunological and, as such, demands the same rigorous control which would be applied to any other immunological investigation.

Thus fluorescent-dye-conjugated-antibody will react specifically with any of its antigen available in tissue and the sites of attachment of the antibody to the tissue will be seen as fluorescent staining, when examined by ultraviolet light. This is the direct fluorescent antibody technique. A modification of this technique

(introduced by Weller and Coons, 1954) is known as the indirect or sandwich technique. In this, the staining process takes place in two stages. First, the immune serum reacts with antigen in the tissue and thereafter the excess serum is removed by washing, leaving the antibody globulin attached to the tissue. In the second stage, the tissue is treated with a fluorescent anti-γ-globulin which stains specifically the antibody attached in the first layer of the sandwich. This technique is much less cumbersome and much more versatile than the direct method, as a single fluorescent anti-γ-globulin serum can be used to test many sera without the necessity of conjugating each serum separately. Furthermore, immunological control can be more vigorously maintained than in the direct fluorescent antibody technique.

The fluorescent antibody technique combines the specificity and sensitivity of an immunological reaction with the anatomical precision of microscopical observation. It is not surprising therefore that it has been applied extensively in the investigation of the cytological localisation of tissue antigens reacting with the autoantibodies which are found in patients with "autoimmune disease". Many of the previous observations with this technique on antinuclear antibodies have not discriminated between different patterns of nuclear staining and so have not exploited the technique to the full.

Since I have noted variations in the patterns of nuclear staining produced by sera containing antinuclear antibodies from different

patients, this thesis will record a critical investigation of the autoimmune antinuclear antibodies by the fluorescent antibody technique.

MATERIALS AND METHODS

Collection and Storage of Patients' Sera The blood samples were allowed to clot, usually at 4°C. but occasionally at room temperature and after clot retraction the serum was centrifuged to remove erythrocytes. Sera was stored frozen at - 20°C mainly in bijou bottles, and larger specimens were split into several containers to avoid unnecessary freezing and thawing.

Preparation of Tissue In the majority of experiments, the source of cell nuclei was rat liver. Rats were stunned, then killed by breaking the neck. Within a few moments of death, small cubes of liver (maximum dimension 3 mm.) were placed on the wall of a thin glass test tube which was plunged into a freezing mixture of acetone and solid carbon dioxide (temperature - 70°C) to produce "snap freezing" of the tissue. The blocks of tissue were then dislodged and frozen on microtome chucks. Sections were cut on a Leitz rotary microtome in a Prentcold cryostat (at the National Institute for Medical Research, Mill Hill) or on a Cambridge rocking microtome in the SIEE cryostat (at the Western Infirmary, Glasgow). The sections were placed on slides and allowed to dry at room temperature. Unless noted specifically in the text, other tissues were snap frozen and sectioned in the same manner.

Blocks could be stored at - 70°C for periods up to 10 days, but when accommodation at this temperature was not available, fresh blocks were obtained from a rat killed on the morning of each experiment. This was necessary as blocks stored at - 20°C showed much more rapid deterioration with growth of ice crystals inside the cell nuclei, loss of certain nuclear antigens and increase in cytoplasmic autofluorescence.

Fluorescent Antibody Staining Technique The "sandwich" fluorescent antibody technique was used in most experiments for the following reasons.

(1) A small number of fluorescent anti-human-γ-globulin sera could be used to test a large number of patients' sera. This saved a great deal of time as direct conjugation is a lengthy process. The clinical surveys reported in Chapter 8 would not have been possible if directly conjugated sera had had to be used. Direct conjugation of a few selected patients' sera was, however, undertaken to confirm the results obtained by the indirect technique.

(2) The "sandwich" technique can be controlled more stringently than the "direct" method (see p. 21).

(3) The "sandwich" technique is more sensitive than the direct technique. The increase in sensitivity has been estimated as 10 x (Coons, 1956) and between 4 x and 12 x (Pressman et al., 1958).

As emphasised above, the sections were always cut from small blocks of tissue. This usually avoided cytological artefacts from

tissue freezing, but when they did occur, the sections were discarded and new blocks of tissue prepared. The use of small blocks also resulted in a considerable economy in patients' sera and fluorescent anti-human-γ-globulin as the volumes required were related to the cube of the diameter of the section which was being stained.

In most experiments, the sections were air-dried on slides but were neither washed nor fixed lest labile or soluble antigens be lost before contact with the patient's serum.

In the first stage of the sandwich staining procedure, the section was treated with one drop of undiluted or diluted patient's serum for 30 min. at room temperature. Drying was prevented by placing the slide in a moist chamber (Petri dishes, 5 in. diameter, with closely fitting lids were used with the slides supported on glass rods over moistened filter paper on the base). During this period any human antibody in the serum would become attached specifically to the corresponding antigen in the section. Most of the serum was removed by flicking the slide which was then washed in barbitone buffered saline (pH 7.2) in a Coplin jar with fairly vigorous shaking (40 - 60 times/min.) for 10 min. on a Griffen and George shaking machine.

The slide was dried with a clean duster to within 1 mm. of the edges of the section. Care was taken to avoid drying before a drop of fluorescein-conjugated anti-human-γ-globulin was applied to the section. The preparation of this reagent is described in Appendix 1/1.

The slide was then placed in the moist chamber for 30 min. at room temperature, to allow specific reaction of the conjugate with any human antibody which had been attached in the first stage. The washing process was then repeated and after drying the section was mounted in buffered glycerol (pH 7.2).

Microscopy The sections were examined under dark-field ultraviolet/blueviolet light with a horizontal optical system of the type described by Young (1961). The light source in the earlier part of the work was a carbon arc lamp but later a high pressure mercury vapour lamp (Mazda ME/D, 250 watt) was used. Primary filtration was achieved by a 2.0 cm. cell containing 10% copper sulphate solution followed by a glass mounted Kodak Wratten No. 50 filter and secondary filtration by a Kodak Wratten gelatin No. 8 filter mounted in the microscope tube. Liquid paraffin or glycerine was used to obtain optical contact between the slide and the Zeiss cardioid dark ground condenser (N.A. 1.05).

Photomicrography The same optical system was used with a camera attached to the microscope tube. A Leica camera body with "Mikas" attachment was used at the National Institute for Medical Research, Mill Hill, and an Edixamat reflex camera at the Western Infirmary, Glasgow. Colour photographs were taken on Kodak 35 mm. High Speed Ektochrome and Monochrome on Adox KB14. Exposures were determined empirically since the light intensities were too low for the exposure meters available.

Immunological Control of Fluorescent Antibody Techniques As has been emphasised above, the fluorescent antibody technique must be controlled like any other immunological reaction if specific staining is to be obtained. In the sandwich fluorescent antibody technique specificity of reaction must be controlled at both the first and the second layers of staining. The analysis of the different serum factors which react with cell nuclei in the first layer of staining is the main topic of this thesis and will be considered in detail in Chapters 2 - 5.

The second layer of the "sandwich" depends on the staining of the attached human γ globulin by fluorescein-conjugated anti-human-γ-globulin. It was essential that this staining should be shown to be specific before the reactions of the patients' sera in the first layer could be investigated. Control was therefore undertaken in three ways. It was shown that:

(1) Staining of human γ globulin attached to the section was inhibited by pre-treatment by unconjugated anti-human-γ-globulin but not by unconjugated anti-human-serum-albumin. Care was taken to demonstrate the "blocking" reaction with an anti-human-γ-globulin from a different animal from that which supplied the serum whose conjugate was being tested. This precaution was necessary because blocking of the action of a conjugate by the unconjugated serum only shows that the same antibody is present in both specimens. The use of sera from two different animals increases the probability that

the reaction is dependent on the principal antibody in the serum, rather than on another contaminating antibody.

(2) Staining of attached human γ globulin was prevented by absorption of fluorescein-conjugated anti-human-γ-globulin with human globulin (isolated with DEAE cellulose chromatography) but was not prevented if absorbed with human serum albumin (Behringwerk, Marburg am Rheim, Germany).

(3) Conjugated anti-human-γ-globulin demonstrated human γ globulin attached to cell nuclei but did not detect human serum albumin at that site (Beck, 1962b).

RESULTS

When human sera were investigated for antinuclear antibodies using the fluorescent antibody technique, it soon became obvious that the patterns of nuclear staining showed great variation when different patients' sera were used. Careful study has, however, suggested that the appearances might be explained in all the cases I have examined by four fundamental patterns of nuclear staining. These patterns may occur singly or in any combination. Examples of sera showing an unmixed pattern of staining were uncommon and one pattern (nucleolar) has not yet been seen alone. Combinations of different patterns were common but such sera commonly showed only one of the fundamental patterns on dilution.

The four patterns of nuclear staining have been described briefly before (Beck, 1961a, 1963) and will now be described in more

detail. These patterns were named "homogeneous", "speckled", "nucleolar" and "membranous". The individual staining patterns have been produced both by the sandwich method and also by directly conjugated patients' sera.

"HOMOGENEOUS" Each nucleus was stained throughout without any diminution in intensity towards the periphery (fig. 1/1). Within the nucleus there were often a few irregular large areas of somewhat more intense fluorescence, similar to the chromatin pattern of the nucleus which can be seen on phase contrast microscopy. There was no accentuation of staining at the nuclear membrane and characteristically the nucleoli were unstained. All nuclei stained with the same intensity.

"SPECKLED" The nucleus showed numerous minute uniform points of fluorescence scattered throughout its substance (fig. 1/2). Because the speckles were sparse at the periphery of the nucleus, its margins were indistinct. The speckles were readily distinguishable from the areas of brighter fluorescence seen in the "homogeneous" pattern of staining, as the latter were irregular in outline and appreciably larger.

"NUCLEOLAR" Each nucleolus was uniformly stained throughout and its smooth surface was sharply demarcated from the adjacent nucleus, so that there was no question of staining of a covering zone of heterochromatin (fig. 1/3). As was usual in adult rat liver cells, one to four nucleoli were stained in each nucleus. There was

considerable variation in nucleolar size from nucleus to nucleus, but when the number of nucleoli in a nucleus was small, the individual nucleoli were commonly larger. This pattern of staining has not yet been encountered alone but has always been associated with staining of one of the other patterns (Beck et al., 1962).

"MEMBRANOUS" Individual nuclei in the section were stained with varying intensity (fig. 1/4). Most of the duller staining nuclei were situated below the surface of the section. Almost all nuclei showed a narrow band of brighter staining at the edge. The heterochromatin was prominently stained around the nucleolus which was unstained. Exuded nuclear material from damaged nuclei was stained as a halo or irregular protruding strands.

DISCUSSION

Validity of the Separation of Different Patterns of Autoimmune Nuclear Fluorescence.

These observations have demonstrated that γ globulins from different patients' sera combine with different parts of the cell nucleus, as the second layer of the sandwich staining will demonstrate only attached human γ globulin (see specificity controls above). Such variations in the sites attachment of human γ globulin to cell nuclei could result in three ways:

(1) The nuclei might have been accidentally modified (e.g. by trauma or drying) before staining was commenced. If this were so, variations in staining patterns would not have any immunological significance.

(2) Different sera might have contained antibodies which reacted with different nuclear constituents, each of which had a different cytological localisation within the nucleus.

(3) Certain patients' sera might have contained non-immunological factors, such as enzymes, which could cause various modifications in the structure of the nucleus; thus, a single antinuclear antibody could produce different patterns of staining.

It is undoubtedly true that trauma can produce marked changes in the pattern of nuclear staining and similar artefacts can follow unsatisfactory snap freezing or prolonged storage of tissue under unsuitable conditions. However, the characteristic variations in staining patterns were found consistently in apparently well processed tissue. I consider that the variations in nuclear staining patterns are the result of serum factors and are not due to accidental changes in the test tissue section for the following reasons:

(a) Any "monospecific" serum regularly produced the same pattern of staining when tested at different times and on sections cut from the livers of different rats.

(b) On dilution of the patient's serum, the intensity of staining was diminished, but the pattern did not alter.

(c) If sections were modified by physical or chemical means, the capacity to be stained by certain sera might be lost whereas the staining properties of other sera were unchanged. Thus, sections prepared from tissue by the freeze substitution method (Balfour, 1961)

could be stained readily by "homogeneous" sera but could not be stained by "speckled" sera.

(d) Extraction of the section, e.g. with 0.15M-NaCl would completely prevent staining by "speckled" sera, but this would not affect staining by "homogeneous" sera (Chapter 3).

(e) When sera of the different types were used to stain tissue from different species, some sera such as the "homogeneous" group stained the nuclei of all the vertebrate tissues examined (Chapter 2), whereas the "speckled" sera stained the mammalian nuclei only and failed to stain frog nuclei (Chapter 3).

(f) A few sera were directly conjugated with fluorescein. These sera produced the same nuclear staining patterns in both the direct and "sandwich" techniques (Chapters 2, 3 and 4).

The variation in the patterns of nuclear staining produced by different sera cannot be due to modification of the test nuclei by a serum enzyme, because:

(i) 7.0S and 19.0S fractions prepared from sera by sucrose density gradient ultra-centrifugation and their γ globulin fractions prepared by DEAE Sephadex chromatography produced the characteristic staining patterns of the corresponding whole sera (Chapter 6). Such fractions would be free from serum enzymes.

(ii) Certain physical and chemical treatments destroy the affinity of nuclei for certain antinuclear sera but not for

others. These findings are incompatible with the hypothesis of serum enzymes modifying the reaction of a single antinuclear antibody.

It is thus certain that different antinuclear sera produce different patterns of nuclear staining because they contain γ globulins with affinity for different nuclear constituents. This is supported by the evidence given in Chapters 2, 3, 4 and 5 on identification of the corresponding antigens. On reflection, it is not particularly surprising that different constituents of the nucleus may have different cytological localisation. The recognition of new and unexpected antibody systems through differences in fluorescent antibody staining patterns has a precedent from the observation by Balfour et al. (1961) who have shown in fluorescent antibody studies on the reaction of Hashimoto's disease sera with sections of human thyroid that there are two distinct staining patterns in the colloid corresponding to two different antibody systems.

Choice of Tissue for the Detection of Differences in Staining Pattern

The different staining patterns can be recognised in most tissues. Although the original choice of rat liver as substrate was fortuitous, this tissue has proved very suitable as the nuclei are large, contain prominent nucleoli and are widely separated by the abundant cytoplasm of the hepatic cells. Thus, analysis of the distribution of staining within the nucleus is easier in liver than in a tissue where the nuclei are small and crowded together as in the normal human thyroid.

The original decision to use unfixed sections in this investigation was also fortunate as the differences in staining pattern produced by different antinuclear sera were much less pronounced after fixation because of the clumping of the nuclear constituents. Thus, White et al. (1961) who fixed their sections failed to appreciate any difference in the patterns of staining shown in their figures 1 and 2, although in retrospect these appear to show the appearances of "speckled" and "homogeneous" staining respectively, with some coarsening of the pattern in the former and some clumping in the latter.

Prior to my first publication on this subject (Beck, 1961b) there had been many investigations of the antinuclear factors by the fluorescent antibody technique, but in none of them had the difference in the staining patterns been appreciated. However, Lachmann and Kunkel (1961) have since confirmed the "homogeneous" and "speckled" patterns. Fennel et al. (1962) have clearly illustrated and differentiated the "homogeneous", "nucleolar" and "membrane" patterns, but they failed to appreciate the immunological significance of their observations. There have also been brief unillustrated reports noting the "nucleolar" pattern (Bardawil et al., 1962) and the "speckled" pattern (Kratchko, 1961) and differentiating between "homogeneous" and "membranous" staining (Casals et al., 1962).

It is not surprising that the different patterns of nuclear staining produced in fluorescent antibody tests for antinuclear

antibodies were not recognised in some of the earlier investigations because the test systems used were often unsatisfactory.

The main technical defects have been:

(a) Absence of certain antigens from the nuclei in the test tissue In some investigations, fowl (Widelock et al., 1961; Thompson, 1962) or human (Calabresi et al., 1959; Alexander et al., 1960) blood films have been used as substrate tissue. The choice of these tissues was unfortunate because the "speckled" and "nucleolar" antigens are absent from the nuclei of fowl erythrocytes and human polymorphonuclear leucocytes (Chapter II). Furthermore, although all the antigens are present in human lymphocytes, the nuclei of these cells are small and clumped and it is very difficult to distinguish different staining patterns unless an oil-immersion objective is used. The staining patterns can be distinguished fairly readily in monocytes, but these cells could be overlooked because they are relatively infrequent in normal blood.

(b) Extraction of antigen from the tissue before staining In other investigations, the test tissues (isolated nuclei, Baugh et al., 1960; human buccal mucosa smears, Rothfield et al., 1961) were treated with saline before staining. As this treatment extracts the "speckled" antigen (and sometimes also the "nucleolar" antigen), these workers would fail to detect the corresponding antibodies.

(c) Tissue fixation has been employed by Weir et al. (1961) and White et al. (1961). The effects of fixation will depend upon the

degree of denaturation produced by its action. If marked, the labile antigens will be destroyed and so the corresponding anti-nuclear antibodies will not be detected in the fluorescent antibody test. If a milder fixative is used, the antigens will probably survive but the nuclear structure will be distorted so that the patterns of staining cannot be distinguished.

SUMMARY

The immunofluorescent staining methods which were used in this investigation were described in detail and the necessity for ensuring adequate immunological control of specificity emphasised.

Considerable variations were noted between the patterns of nuclear staining produced with different human sera in the fluorescent antibody test for antinuclear antibody. It was suggested that all appearances could be explained by four fundamental patterns which could occur singly or in any combination. The patterns, "homogeneous", "speckled", "nucleolar" and "membranous" were described and illustrated.

Evidence was given to show that each staining pattern was produced by an antibody to a different constituent of the nucleus.

PART II

(Chapters 2, 3, 4, 5 and 6)

THE IMMUNOLOGY OF HUMAN ANTINUCLEAR ANTIBODIES

The investigations on the nature of the antigens with which human antinuclear sera react are described in Chapters 2 - 5, whilst the experiments which demonstrated that the corresponding serum factors are antibodies are recorded in Chapter 6.

Chapters 2, 3, 4 and 5 will be concerned separately with the antigens which react with sera producing the four basic patterns of nuclear staining in the fluorescent antibody technique. As the approach has been similar in each case, the basic experimental techniques will be described here to avoid repetition in the individual chapters.

MATERIALS AND METHODS

(A) The Nuclei of Cells from Different Organs in a Wide Variety of Animal Species

These were stained in the fluorescent antibody technique by the different types of antinuclear sera so that the organ and species distribution of the corresponding antigens could be determined. It was hoped that any differences in the distribution of antigens might be related to known differences in the chemical composition of these nuclei.

Table 2/1 shows the different varieties of cell nuclei which have been used as substrate for reaction with human antinuclear sera. Unfixed cryostat sections were obtained from snap-frozen blocks of all the solid tissues except rat testis where impression smears (both unfixed and after brief fixation in absolute acetone) were used.

The HeLa cells, chick fibroblasts and monkey kidney cells were grown in tissue culture on coverslips and were used after fixation in absolute acetone for 3 - 5 sec. Human blood and various protozoa were prepared as air-dried smears on chemically clean slides without fixation. Human buccal mucosal cells and spermatozoa from various mammalian species were fixed in absolute acetone before use as antigen.

These antigen preparations were stained by the "sandwich" fluorescent antibody technique described in Chapter 1; the specificity of staining by the fluorescein-conjugated rabbit anti-human-γ-globulin was controlled as described in that Chapter.

(B) Staining of modified cell nuclei

In these experiments, the cell nuclei were subjected to certain standard histochemical procedures before treatment with the different types of antinuclear sera in order to determine the solubility and stability of the antigen and its resistance or susceptibility to enzymatic digestion. Cryostat sections of fresh-frozen rat liver were always used as substrate and the histochemical techniques employed were:-

(1) Extraction Sections were covered with several drops of solvent (distilled water, 0.15M-NaCl or 1.0M-NaCl) and placed in a moist chamber for periods of ½ to 2 hr at 4°C, room temperature or 37°C. Thereafter, the sections were stained in the usual manner.

(2) Antigen Stability Sections were fixed in absolute ethanol or

acetone for 10 min. at room temperature or for periods up to 1 hr at - 20°C, in 70% ethanol or 4% aqueous formaldehyde at room temperature or in absolute methanol at - 20°C. After washing in barbitone buffered saline (pH 7.4) the sections were stained in the usual manner.

(3) Enzymic digestion Sections were covered by several drops of enzyme solution or the appropriate buffer control and placed in a moist chamber at 37°C for 30 min. After washing in barbitone buffered saline (pH 7.4) the sections were stained in the usual manner. Fresh-frozen rat liver sections, both unfixed and fixed in acetone for 10 min. at room temperature, were suitable for DNase and RNase digestion experiments. Unfixed sections were however unsatisfactory for digestion by the proteolytic enzymes as the sections usually floated off the slides during staining procedure, but prior fixation in acetone (10 min. at room temperature) usually ensured that the sections adhered to the slide.

DNase (0.1 mg./ml.) was dissolved in barbitone-buffered 0.15M-NaCl (pH 7.4) containing 40 µg Mg^{++}/ml., while RNase (1.0 mg./ml.) was used in ion exchanged water. Trypsin (5.0 mg./ml.) and chymotrypsin (5.0 mg./ml.) were used in barbitone buffered 0.15M-NaCl (pH 8.0) and pepsin was used in 0.15M-NaCl (pH 2.0). The enzyme preparations were supplied by the Worthington Biochemical Corporation, Inc.

(C) Staining of Isolated Tissue Components Dried as Films on Slide (the fluorescent spot test).

An attempt was made to investigate the nuclear antigens involved in fluorescent antibody staining with antinuclear sera by using a completely *in vitro* system. Smears of a variety of purified tissue extracts were air-dried at room temperature on acid-cleaned microscope slides and fixed in absolute acetone for 10 minutes at room temperature. The smears were then stained by human antinuclear sera followed by fluorescein-conjugated anti-human-γ-globulin from which any non-specific staining had been removed by the appropriate absorptions. The following purified tissue constituents were investigated:-

(i) Calf thymus nucleohistone extracted by both the low and high ionic strength methods (Chargaff, 1955).

(ii) Calf thymus DNA (Worthington Biochemical Corporation, Inc.)

(iii) Calf thymus histone (Worthington Biochemical Corporation, Inc.)

(iv) Calf liver histone prepared by the method of Monty and Dounce (1959).

(v) Calf liver "nuclear globulins" prepared by the method of Monty and Dounce (1959).

(vi) Rat liver saline soluble nuclear proteins prepared by my own method (Beck, 1962a).

(vii) Cytoplasmic RNA extracted from mouse liver microsomes by Miss B. A. Askonas, National Institute for Medical Research, London.

(D) Absorption Studies on Antinuclear Sera

The sera were tested at a concentration 100 times greater than the end point in titration. They were absorbed for 1 hr at 37°C or 18 hr at 4°C with an equal volume of various tissue preparations. After centrifugation, the absorbed sera were titrated to determine whether there had been any loss of titre.

The following preparations were tested:-

(i) Rat liver nuclei isolated in 0.22M sucrose by the method of Chargaff (1955).

(ii) Rat liver mitochondria and microsomes isolated by the method of Hogeboom and Schneider (1955).

(iii) Calf thymus nucleohistone prepared by the low and high ionic strength methods (Chargaff, 1955) (10 mg./ml.).

(iv) Calf thymus DNA (Worthington Biochemical Corporation, Inc.) (100 mg./ml.)

(v) Calf thymus histone (Worthington Biochemical Corporation, Inc.) (10 mg./ml.)

(vi) Calf liver histone prepared by the method of Monty and Dounce (1959). (approximately 5 mg./ml.).

(vii) Calf liver "nuclear globulins" prepared by the method of Monty and Dounce (1959) (approximately 5 mg./ml.).

(viii) Rat liver soluble nuclear proteins prepared by my own method (Beck, 1962a) (approximately 5 mg./ml.).

(ix) Mouse liver cytoplasmic RNA prepared by Miss B. A. Askonas, from isolated mouse liver microsomes (5 mg./ml.).

(E) Annulment Experiments

It is well known that pre-treatment with an unconjugated antibody will prevent or greatly diminish the staining of a tissue antigen by a fluorescent antibody, provided the two sera react specifically with the same antigenic determinants, whereas the intensity of staining will not be diminished if the antibodies have different specificities (Nairn, 1962). An attempt was therefore made to confirm the suggestion that different patterns of nuclear staining are caused by antinuclear sera with different immunological specificities (Chapter 1) and to establish that sera producing a given pattern all react with the same antigen by annulment experiments with directly conjugated human antinuclear sera.

Sections (if necessary, fixed to retain antigens) were treated for 30 min. with strong antinuclear sera of various types, washed and then treated with directly conjugated sera. After washing, the sections were mounted and examined in the usual manner. Annulment was considered to have occurred if pre-treatment by a given serum consistently produced gross diminution in the intensity of the staining by the directly conjugated antinuclear serum.

CHAPTER 2

THE "HOMOGENEOUS" ANTIGEN

This is the antigen with which human antinuclear sera most frequently react (Table 8/38). It therefore seemed highly probable at the beginning of this investigation, that this antigen would correspond in its properties to the previous descriptions of the antigen which reacts with sera containing uncharacterised "antinuclear factor". The experiments recorded below have confirmed this supposition.

(A) Organ and Species Distribution of "Homogeneous" Antigen

(A) Organ and Species Distribution of "Homogeneous" Antigen'cus"

antigen was distributed widely in vertebrate tissues and when unfixed cryostat sections were used as substrate, the typical "homogeneous" staining pattern was obtained in cell nuclei from different organs in a wide variety of species. When the cells had been fixed (e.g. tissue culture preparations) or traumatized (e.g. isolated rat liver nuclei) the staining pattern showed a variable degree of coarse granularity. The only nuclei in which the "homogeneous" antigen could not be demonstrated were those of mammalian spermatozoa (confirming Holborow and Weir's observations, 1959) and certain primitive protozoa, nor was the antigen detected in the inclusion bodies of Trachoma virus in the cytoplasm of monkey kidney cells.

Although it is not possible to draw any firm conclusions from

these experiments, it is noteworthy that the antigen was demonstrated in nuclei where DNA is known to be combined as nucleohistone (e.g. all vertebrate somatic cells) and furthermore that the distribution of the "homogeneous" antigen corresponds to the distribution of the DNA-histone complex in the nucleus. Moreover, "homogeneous" antinuclear sera failed to stain DNA-containing structures where the DNA is not complexed with histone, e.g. spermatozoal nuclei (where the DNA is combined as nucleoprotamine, Vendrely et al., 1957) and Trachoma virus (viral DNA is not complexed with protein, Luzzati and Nicolaïeff, 1963); the state of combination of DNA in protozoa has not been investigated previously (Chapter 12).

(B) Reaction of "Homogeneous" Antinuclear Sera with Nuclei modified by Histochemical Procedures

The altered nuclei were stained by three high titre monospecific "homogeneous" antinuclear sera (Dun., McL. and War.).

(1) Extraction Pre-treatment with 0.15M-NaCl (pH 7.4) at 4°C, 15°C and 37°C did not modify the subsequent staining pattern produced by "homogeneous" antinuclear sera, whereas distilled water caused moderate swelling of the nuclei but did not otherwise alter their staining properties. After exposure to 1.0M-NaCl (pH 7.4) the nuclei became markedly swollen and showed numerous broad and filamentous projections and the antigen could be demonstrated both

in the body of the nucleus and in the projections. In other preparations, similar projections were readily stained by haematoxylin or by the Feulgen method. The experiments indicate that the "homogeneous" antigen behaves in the same way as nucleohistone during the extraction procedure.

(2) Stability The intensity of nuclear staining after various fixation procedures is shown in Table 2/2. The greatest damage to the "homogeneous" antigen was produced by 4% aqueous formaldehyde and 70% ethanol. As these fixatives produced protein denaturation but had little effect on DNA, it seemed highly probable that there was a protein component in the "homogeneous" antigen.

As absolute acetone has little effect on the antigen at room temperature, this has been the fixative of choice to destroy cell membrane selective permeability before staining the intact cells in tissue cultures. Absolute ethanol and acetone did not affect the antigen at - 20°C, thus it was not surprising that the "homogeneous" staining pattern was readily demonstrable in sections of guinea pig liver and lymph node prepared by the freeze substitution method (Dalfour, 1961).

(3) Enzymic digestion The results of staining by the "homogeneous" antinuclear antibody after various enzyme treatments are shown in Table 2/3, which shows that staining of nuclei could be prevented by DNase or proteolytic digestion but not by the action of RNase. These experiments suggested either that the "homogeneous" antigen

incorporated DNA and protein or that it was intimately bound to the DNA protein complexes in cell nuclei and confirmed the findings in the other histochemical investigations.

Experiments on the staining properties of modified nuclei had not been attempted previously using sera identified as the "homogeneous" type, nor had there been any previous attempt to investigate the stability of nuclear antigens. My results confirm the effect of extraction by distilled water and 1.0M-NaCl reported by Friou (1958a) who was using sera containing antinuclear antibodies of unidentified type. Susceptibility to DNase digestion has also been reported by Friou (1958a), Holborow (1960) and Weir and Holborow (1962) using sera of unspecified type. Weir and Holborow (1962) have shown that DNase digestion after attachment of human antinuclear antibody will remove the Feulgen stainable material but not the attached human antibody. Although DNA must be present in the nuclei to allow attachment of antinuclear antibody, the antibody will persist even after removal of the DNA; this observation would be compatible with the hypothesis that the "homogeneous" antinuclear antibody reacts with both components of the nucleohistone complex.

(c) The Fluorescent Spot Test

Nucleohistone extracted by both methods was intensively stained by "homogeneous" sera but histone, "nuclear globulins" and saline-soluble nuclear proteins were unstained. The films of DNA did not adhere to the glass during the staining procedure.

These results confirm and elaborate the series of reports by Friou (1958a and b, 1962) in which he has demonstrated that antinuclear sera of unspecified type can stain smears of nucleohistone. The nucleohistone fluorescent spot technique has also been used by Crawford et al., (1959) but their results were not supported by any immunocytochemical search for antinuclear antibodies.

(D) Staining with "Homogeneous" Sera after Absorption with Various Tissue Components

A series of experiments was undertaken to determine whether isolated cellular organelles and purified tissue extracts were capable of absorbing the "homogeneous" antinuclear antibodies from the sera of 6 patients (Dun., Mot., McL., War., Bry. and Pea.). Absorption with isolated rat liver nuclei and calf thymus nucleohistone prepared by either method usually removed the "homogeneous" antinuclear antibody completely and invariably caused a gross diminution of titre. Absorption with histone did not affect the titre provided that the system was adequately buffered between pH 7.0 and 7.4. None of the other samples tested absorbed the "homogeneous" antibody.

Lachmann and Kunkel (1961) have confirmed my report (Beck, 1961a) that the "homogeneous" antinuclear antibody can be removed from serum by absorption with nucleohistone. Friou (1958b) using sera of unspecified type has shown absorption of antinuclear antibodies from three sera by whole nuclei and isolated nucleohistone, while Weir and Holborow (1962) have shown that 21 of 24 sera containing

antinuclear factor can be absorbed by nucleohistone. It is interesting that Friou (1958b) and Weir and Holborow (1962) should show that a high proportion of antinuclear sera can be absorbed by nucleohistone specifically while I have shown that the "homogeneous" antibody is the type most commonly encountered in human disease (Chapter 8) and that sera of this type are also absorbed specifically by nucleohistone.

(E) Annulment Experiments

An attempt was made to annul staining by directly conjugated Dun. (a strong "homogeneous" serum) by pretreatment with 6 "homogeneous" sera (Dun., Mot., McL., War., Bry. and Pea.) and 6 "speckled" sera (Tre., Plu., Ral., McD., McLau. and Nis.). All 6 "homogeneous" sera annulled subsequent staining by fluorescein-conjugated Dun. serum completely or partially, whereas none of the "speckled" sera were effective. This has been confirmed by Lachmann and Kunkel (1961).

The experimental evidence presented above can be summarised thus:

1. The "homogeneous" antigen was present at sites where nucleohistone is found and was absent where DNA is either free or combined with protamine.

2. The solubility and stability of the "homogeneous" antigen were closely similar to those of nucleohistone.

3. The susceptibility of the "homogeneous" antigen to enzymic digestion was similar to that of nucleohistone.

4. Of the cell extracts investigated by the fluorescent spot test,

only nucleohistone was stained by "homogeneous" antinuclear sera.

5. The "homogeneous" antinuclear antibody could be absorbed from patients' sera by whole nuclei and nucleohistone but not by any other tissue component.

6. Annulment experiments showed that all the "homogeneous" antinuclear sera tested reacted with the same antigenic determinant.

DISCUSSION

The similarity between the characteristics of the "homogeneous" antigen and nucleohistone was remarkable. As the properties have been investigated by many different techniques, it was highly improbable that any contaminating substance could have been associated with nucleohistone under so many varying conditions, and yet not have been recognised previously. The whole weight of evidence therefore proves that the "homogeneous" antigen was nucleohistone.

Antinucleohistone is the antinuclear antibody which has been investigated most widely and it had been demonstrated previously in the serum of patients with "connective tissue" diseases by a variety of techniques, namely:

(a) Complement fixation (Christian et al., 1958; Fessel et al., 1959; Holman et al., 1959; Scalletar et al., 1960; Edington and Walford, 1961; Rothfield et al., 1961; Tomes et al., 1963a).

(b) Precipitation reactions (Holman and Kunkel, 1957; Holman and Deicher, 1959).

(c) Latex agglutination (Christian et al., 1958; Willkens et al., 1958; Dubois et al., 1961).

(d) Tanned red cell agglutination (Miescher and Strässle, 1957; Goodman et al., 1960).

(e) Immuno-electrophoresis of nuclear extracts (Atchley, 1961a and b).

(f) Fluorescent spot technique and modifications (Friou, 1957, 1958a and b, 1962; Crawford et al., 1959).

(g) Nuclear agglutination (van Soren, 1961).

Furthermore, it has been shown that the L. E. cell phenomenon depends upon the presence of anti-nucleohistone in the serum (Holman and Kunkel, 1957; Holman and Deicher, 1959; Lachmann, 1961b), although this test also requires a phagocytosis promoting factor which is probably complement (Aisenberg, 1959; Lachmann, 1961a). The "homogeneous" antinuclear antibody has been found in the serum of all "connective tissue" disease patients who had a positive L. E. cell test (Chapter 8).

The antigenic determinants in nucleohistone

The nature of these antigenic determinants is not yet known. However, Friou, (1958b) has shown that antinuclear antibody has somewhat less affinity for artificial DNA protein complexes than for extracted nucleohistone, and Lachmann (1961b) has demonstrated that the L. E. factor is much more rapidly absorbed by water-extracted nucleohistone than by 1.0M-NaCl extracted nucleohistone, which is thought to be a reassociation chemical artefact (Chargaff, 1955; Zubay and Doty, 1959). Edington and Walford (1961) using a comple-

ment fixation test claim that lupus erythematous sera have greater affinity for non-fibrous nucleohistone than for fibrous nucleohistone, which they consider to be altered from its native state. As the less denatured forms of nucleohistone show greater affinity for anti-nucleohistone, consideration of the structure of nucleohistone may give some indication of the type of antigenic determinants which might be involved.

An area on the surface of a molecule can act as an antigenic determinant only if it has polar groups which are held sterically rigid. Estimates of the size of such antigenic determinants have varied from about 1,000 sq.Å (Landsteiner, 1945) to about 6 hexose residues (Kabat, 1954).

There is now considerable evidence that native DNA consists of a double helix of polynucleotide chains with bases directed towards the axis where they are held together by hydrogen bonds (Watson and Crick, 1953a and b) while in nucleohistone the histone is arranged in the broad groove of the DNA helix to form a triple helix (Zubay and Doty, 1959). The exposed part of the DNA molecule is formed of a regular rigid sugar-phosphate chain and this should not show organ or species specificity. The histone polypeptide chain is extended and held by hydrogen bonds in the broad groove of the DNA helix, so that the exposed part will probably consist mainly of the "backbone" of the molecule and it is probable that short amino-acid sequences will be common to all histones despite the considerable variations in the overall composition of histones

isolated from different sources.

It would therefore be theoretically possible for a small area on the surface of a nucleohistone molecule involving both DNA and histone moieties to act as an antigenic determinant as it would have polar groups from both the sugar-phosphate chain and the polypeptide chain held sterically rigid. It is highly probable that such areas would not show either species or organ specificity.

SUMMARY

The experiments reported in this Chapter have shown that the "homogeneous" pattern of nuclear staining is produced by sera containing anti-nucleohistone and that antigenicity depends upon DNA combined with histone.

CHAPTER 3

THE "SPECKLED" ANTIGEN

The "speckled" pattern of nuclear staining was the second commonest abnormality detected in the fluorescent antibody test for antinuclear antibodies (Table 8/38). The antigen involved in this staining reaction was investigated in a similar manner to that used for the "homogeneous" antigen. Since the "speckled" antigen did not correspond to any well recognised nuclear constituent, the products of various purification procedures were tested for antigen activity by a series of absorption experiments and the antigen was identified as a previously unrecognised protein of the cell nucleus.

(A) Organ and Species Distribution of the "Speckled" Antigen

The findings are summarised in Table 3/1. The "speckled" antigen was distributed widely in mammalian tissues. When unfixed cryostat sections were used as substrate the typical "speckled" staining pattern was seen in cell nuclei from different organs in a wide variety of species. If, however, the cells had been fixed (e.g. tissue culture preparations) or traumatised (e.g. isolated rat liver nuclei) the staining pattern showed a variable degree of course granularity, approximating to that seen with the "homogeneous" system on fixed tissues. The only mammalian nuclei in which the "speckled" system could not be detected were spermatozoa and polymorphonuclear leucocytes (Chapter 11). The "speckled" antigen

was not demonstrated in any sub-mammalian species.

The observed distribution of the "speckled" antigen does not correspond to that of either DNA or histone and it is therefore probable that the "speckled" antinuclear sera are reacting with some other antigenic determinant common to most mammalian somatic cell nuclei. It is however, realised that the "taxonomic" observations have not eliminated the possibility that the "speckled" antigen is a mammalian-specific determinant on DNA, histone or nucleohistone.

(B) Reaction of "Speckled" Antinuclear Sera with Nuclei modified by Histochemical Procedures

The altered nuclei were stained by 3 high titre monospecific "speckled" antinuclear sera (Tra., Ral. and Plu.).

(1) Extraction Treatment of cryostat sections of rat liver with distilled water or 0.25M sucrose in ion-exchanged water did not alter the subsequent staining pattern produced by "speckled" sera but exposure to 0.15M-NaCl (pH 7.4; temperature 4°C to 37°C) or 1.0M-NaCl for as short a time as 5 min. prevented staining by the "speckled" antibody. In view of the rapidity of the process and the absence of temperature dependence, it was probable that this represented extraction by the sodium chloride solution, but it was also possible that the antigen could have been destroyed by sodium chloride or that sodium chloride might have activated a tissue enzyme which could destroy the "speckled" antigen.

(2) Stability of the antigen in sections The intensity of nuclear staining after various fixation procedures is shown in Table 3/2. The "speckled" antigen was readily destroyed by 4% aqueous formaldehyde or 70% ethanol and to a lesser extent by absolute ethanol. Although much more labile to chemical fixation than the "homogeneous" antigen (Table 2/2), the "speckled" antigen was also destroyed preferentially by protein denaturing agents: this suggested that the antigen might contain protein.

As absolute acetone has little effect on the antigen at room temperature, this has been the fixative of choice for destruction of cell membrane selective permeability before staining the intact cells in tissue cultures. Absolute ethanol damaged the antigen even at - 20°C and had an even more deleterious effect at room temperature, thus it was not surprising that the "speckled" antigen could not be demonstrated in sections of guinea pig liver and lymph node prepared by the freeze-substitution method (Balfour, 1961).

Lachmann and Kunkel (1961) have shown that 0.01M sodium periodate treatment can destroy the "speckled" antigen in acetone-fixed tissue sections but does not damage the "homogeneous" antigen. I have confirmed these findings but found that the preparations were difficult to interpret because of the marked increase in tissue autofluorescence induced by exposure to periodate.

(3) Enzymic digestion The results of staining by "speckled" antinuclear antibodies after various enzyme treatments are given in

Table 3/3, which shows that neither DNase nor RNase damaged the antigen. The proteases prevented subsequent staining; this suggested that the "speckled" antigen might have a protein component, but these results must be interpreted with caution in view of the gross cytological damage produced by these enzymes.

(4) Saline treatment of isolated nuclei Nuclei were isolated from rat liver after homogenisation in 0.25M and 2.2M sucrose in ion-exchanged water following closely the techniques described by Dounce (1955) and Chauveau et al. (1956) respectively. Freshly isolated nuclei in sucrose suspending medium were treated for 30 min. with "speckled" sera diluted at least ten times in the corresponding sucrose medium, washed three times in saline, collected by centrifugation and treated for 30 min. with a fluorescein-conjugated rabbit anti-human-γ-globulin which did not itself stain isolated nuclei. The nuclei were washed three times in saline, collected by centrifugation, mounted in buffered glycerol and examined in the usual ultraviolet/blue violet optical system. Other preparations of isolated nuclei were extracted with barbitone buffered 0.15M-NaCl (pH 7.4) before staining by the "speckled" antibody. In concurrent experiments, unextracted and buffered saline extracted nuclei were stained with the normal and "homogeneous" sera.

Table 3/4 shows that the nuclear constituent reacting with the "speckled" antibody was removed by treatment with buffered saline for 5 min. whereas the "homogeneous" antigen (nucleohistone) was not

affected even after extraction periods up to 3 hr. Examples of staining of unextracted nuclei by sera Tra. ("speckled"), Dun. ("homogeneous") and Bec. (normal) are shown in Figs. 3/1-3. This confirms the observations made on frozen sections.

(C) The Fluorescent Spot Test

Attempts were made to stain dried films of the various tissue constituents listed on p. 39 with "speckled" antinuclear sera, but in no case was specific staining obtained.

(D) Staining with "Speckled" Sera after Absorption with Various Tissue Constituents

These experiments were carried out in a similar manner to that used in the investigation of the "homogeneous" antigen using a panel of 6 "speckled" sera (Tre., Plu., Ral., Nis., McDon. and McLau.). The "speckled" antibody was not absorbed by rat liver mitochondria or microsomes, calf thymus nucleohistone, commercial DNA or histone, calf liver histone or nuclear globulins or mouse liver cytoplasmic RNA.

As rat liver nuclei suspended in the original isolating medium (0.25M sucrose) were capable of absorbing the "speckled" antinuclear antibody, whereas saline washed nuclei were not. Experiments were undertaken to determine whether saline treatment extracted the "speckled" antigen: large bulks of nuclei were required and I found that these could be prepared most conveniently from groups of 6 rat livers by the method described by Chauveau et al. (1956).

Isolated nuclei were stirred for 3 hr at 4°C with 10 volumes of 0.15M barbitone buffered saline (pH 7.4) and after centrifugation the supernatant was concentrated by pressure dialysis to the original volume of the isolated nuclei. The extracts were tested for the presence of the "speckled" antigen by absorption tests in which 3 volumes of the extract were incubated at 37°C for 1 hr with 1 volume of a "speckled" serum diluted to 1/10th of its final titre. The content of "speckled" antigen in the extract was estimated by determining the maximum dilution of the extract which would completely absorb the standard "speckled" antinuclear antibody preparation. Eleven saline extracts of nuclei were prepared and all would absorb the "speckled" antibody, some were effective even when diluted sixteenfold. Such extracts did not absorb "homogeneous" or "nucleolar" sera.

An attempt was next made to purify the "speckled" antigen using the scheme shown in Fig. 3/4. The saline extract was acidified to pH 4.5 for 15 min. and then returned to pH 7.2. Thereafter, the extract was dialysed against 0.01M-NaCl overnight at 4°C. Any precipitate apparent after each manipulation was removed by centrifugation and resuspended in half the starting volume of barbitone buffered 0.15M-NaCl (pH 7.4). Samples of the individual fractions and of the resuspended precipitants were tested for antigen activity as described above and their ultraviolet absorption spectra were determined on a Unicam SP 500 spectrophotometer. The final

supernatants showed no loss of "speckled" antigen and the precipitates were inactive.

During purification, the ratio of the optical density at 260 mμ to that at 280 mμ diminished, indicating that the "speckled" antigen is not a nucleic acid. The absorption spectrum of one final supernate is shown in Fig. 3/5 and the others were similar. These absorption spectra show that there was a high protein and low nucleic acid content in such preparations.

In the purified extracts, the "speckled" antigen was stable to freezing and thawing and it has been stored at - 10°C for 6 months without apparent loss of activity; all activity was lost after heating to 56°C for 30 min. Within the temperature range - 10°C to + 15°C the antigen preparations are stable in 0.15M-NaCl between pH 1.0 and pH 8.0.

The effect of various oxidising agents on purified extracts of the "speckled" antigen has been investigated in view of the suggestion from its lability in neutral periodate, that this antigen may be a glycoprotein (Lachmann and Kunkel, 1961). "Speckled" antigen preparations were treated with 0.005M concentrations of various oxidising agents for 2 hr at room temperature, and then further action was stopped by the addition of 33% glycerol in buffered saline (pH 7.2) for 2 hr at room temperature. The antigen activity was then tested as described above. Under these conditions the "speckled" antigen was completely destroyed by potassium

permanganate and potassium dichromate, partly destroyed by sodium periodate and sodium iodate, but unaffected by potassium ferricyanide or hydrogen peroxide. As controls, the oxidising agents were mixed with glycerol before addition to the "speckled" antigen solution; when this was done, the antigen retained its full activity.

In an attempt to determine the sedimentation coefficient of the "speckled" antigen, various purified extracts were centrifuged by Dr P. A. Charlwood of the National Institute for Medical Research, Mill Hill, in a sucrose density gradient in a preparative centrifuge (model E Spinco) with addition of trace amounts of markers (radioactive iodine labelled proteins of known sedimentation coefficients, Charlwood, 1963). The marker protein was traced in the individual fractions by counting in a scintillation counter. Each fraction was tested in titration to determine the lowest concentration which would annul completely the standard "speckled" antibody preparations. By comparison in this way, the "speckled" antibody was shown to have a much lower sedimentation coefficient than human 7.0S γ globulin or bovine serum albumin (4.0S) but only a little lower than ovalbumin (3.5S) as shown in Table 3/5. The only available purified protein with a sedimentation coefficient lower than 3.5S was lyszozyme (Park Davis and Company) but attempts to use this as a marker were unsuccessful because the activity of the "speckled" antigen was lost during the fractionation procedure with this enzyme preparation.

Attempts to demonstrate the reaction of purified "speckled"

~~antibody~~ antigen preparations with "speckled" antinuclear sera by precipitation reactions in agar or by immuno-electrophoresis in agar have been unsuccessful.

(E) Annulment Experiments

Annulment experiments were carried out using a technique similar to that described on p. 41 except that acetone-fixed cryostat sections of rat liver were used because of the high solubility of the "speckled" antigen. Pre-treatment by any of 6 "speckled" antinuclear sera (Tra., Plu., Ral., McD., McLau. and Nis.) prevented staining by directly conjugated serum Tra. (a strong "speckled" serum) but none of 6 "homogeneous" antinuclear sera (Dun., Mot., McL., War., Bry. and Pea.) were effective.

DISCUSSION

The results of the absorption experiments showed that the "speckled" antigen was a saline-soluble protein with sedimentation coefficient less than 3.6S and that although stable to freezing and thawing, it is destroyed by heating to 56°C for 30 min. Study of tissue sections confirms its free solubility in physiological saline and the enzyme tests confirm that it is a protein. This antigen was found in the nuclei of all mammalian somatic cells except mature polymorphs.

Antibodies to a phosphate-buffer extract of isolated nuclei have been demonstrated previously by a complement-fixing technique (Holman et al., 1959) and it seems likely that this antigen is the

same as the "speckled" antigen as Lachmann and Kunkel (1961) have shown that a few sera with high titre complement fixing antibodies against a phosphate buffer extract of nuclei also showed "speckled" nuclear staining pattern in the fluorescent antibody technique.

Holmen (1961) and Lachmann and Kunkel (1961) have suggested that the antigen is a glycoprotein since it was readily destroyed by periodate which selectively destroyed glycollic groupings. My experiments showed that the action of periodate was similar to that of other oxidising agents and so it was unlikely that carbohydrate forms a significant part of the "speckled" antigen molecule.

The relationship of the "speckled" antigen to the structure of the nucleus. In fixed preparations, the precipitated strands of chromatin are separated by a poorly stained material which has been called nuclear sap, but until recently, there has been no certainty that this is not a fixation artefact. In living cells, the nuclei appear much more "homogeneous", but microdissection studies on tissue culture cells have suggested that there may well be a low viscosity fluid phase in addition to the well recognised viscous nucleoprotein phase (Chambers and Fell, 1931). Claude (1943) has since shown by ultracentrifugation of whole liver cells that the chromatin and nucleoli can be sedimented leaving a supernatant of nuclear sap in the upper pole of the nucleus. There have been a few cytochemical investigations on the nature of the nuclear sap (see Brachet, 1957) but Brown et al. (1950) have isolated nuclear sap from frog oocytes

and shown by direct biochemical analysis, that it contains a saline-soluble protein with an ultraviolet absorption spectrum similar to that of the purified "speckled" antigen described above.

There have been few investigations of the proteins which can be extracted from isolated nuclei by solvents. Barton (1962) has determined the electrophoretic mobility of protein extracted by 0.15M-NaCl from rat liver nuclei isolated in sucrose and in a more recent communication he has shown by ultracentrifugation that part of the protein had a sedimentation coefficient of 3.0S to 4.0S whereas the remainder is 7.0S to 8.0S (Barton, 1962). Frenster et al. (1960) have shown that 0.1M-NaCl extract of thymus nuclei contain sedimentable ribonucleoprotein and a soluble protein and Wang (1961) has shown a soluble protein with a sedimentation coefficient of 2.5S in these extracts. The results of these investigations must be considered carefully as Dounce (1955) has emphasised that nuclear constituents may be lost to the suspending fluid during isolation whereas cytoplasmic constituents may be absorbed or combined with DNA and, moreover, a preparation of isolated nuclei may well be contaminated with cytoplasmic organelles.

Nevertheless, the properties of these biochemically isolated soluble proteins have been remarkably similar to those of the "speckled" antigen. There can be little doubt that the "speckled" antigen is a true constituent in interphase nuclei since it is highly improbable that a cytoplasmic antigen could move into and

become adsorbed on the nuclei during snap freezing or during fixation of monolayer tissue cultures as both processes will stop, almost instantaneously, the movement of substances within the cell. Thus at least one (the "speckled" antigen) of the saline-soluble proteins which have been extracted from isolated nuclei must be a true constituent of these nuclei *in vivo*.

The metabolic significance of the "speckled" antigen and the other soluble nuclear proteins has not yet been determined but it would be interesting to investigate the enzymic activity of the purified preparations of the "speckled" antigen. As a complementary investigation, it would be interesting to trace enzymes suspected of nuclear localisation by fluorescent experimentally induced antibodies but this will become possible only when highly purified preparations of the enzymes become available for use as antigens.

SUMMARY

The "speckled" antigen has been identified as a saline-soluble protein of the nuclear sap. It appears to be identical with the phosphate buffer extract antigen. Its localisation in the nucleus proves that at least one of the soluble proteins extracted biochemically from isolated nuclei is a true constituent of the nucleus.

CHAPTER 4

THE "NUCLEOLAR" ANTIGEN

The nucleolar pattern of nuclear staining was uncommon and has been encountered with only 18 of the sera studied in this series (Table 3/38). In each case, the serum has contained in addition either "homogeneous" or "speckled" antinuclear antibody, usually in much lower titre than the antinuclear antibody. Only 2 sera (Mui. and Scr.) have proved suitable for investigation of the nucleolar antigen, because these were the only antinucleolar sera available in adequate quantity which contained high titre antinucleolar antibody, yet were contaminated with relatively low titre "homogeneous" or "speckled" antinuclear antibody respectively. After absorption with either calf thymus nucleohistone or purified rat liver "speckled" antigen respectively, these sera gave nucleolar staining only. The absorbed preparations were used in the investigations on the nature of the nucleolar antigen and will be designated MUI. and SCR. in this chapter.

(A) Organ and Species Distribution of the "Nucleolar" Antigen

The findings are summarised in Table 4/1. Nucleoli were stained in all types of vertebrate somatic cell nuclei studied except normal mature human polymorphonuclear leucocytes or chronic myeloid leukaemia stab cells and polymorphonuclear leucocytes (Chapter 11). In all cases the appearances of nucleoli stained with the antinuclear

antibody were similar to those seen in haemalum and eosin preparations. Nucleolar antigen was not detected in mammalian spermatozoa or in unicellular flagellates (Chapter 12). Neither MUI. nor SCR. stained cytoplasmic organelles rich in RNA (e.g. Nissl's substance in cortical neurones).

The antinucleolar antibody appeared to react specifically with vertebrate nucleoli. It was noteworthy that it does not react with the RNA granules in protozoa nor with vertebrate cytoplasmic RNA, but no conclusions can be drawn from these findings as it is well known that nucleolar RNA is chemically different from the RNA in other parts of the cell (Sirlin, 1962).

(B) Reaction of Antinucleolar Sera with Nuclei modified by Histochemical Procedures

The altered nuclei were stained by MUI. and SCR.

(1) Extraction Treatment of cryostat sections of rat liver with distilled water or 0.25M sucrose in ion-exchanged water did not alter the subsequent staining pattern produced by antinucleolar sera MUI. or SCR. but exposure to 0.15M-NaCl (pH 7.4; temperature 4°C to 37°C) or 1.0M-NaCl prevented staining. The action of saline on nucleoli was considerably less rapid than its action on the "speckled" antigen (p. 53), but in view of the lack of temperature dependence, it was probable that this action represented extraction of the nucleolar antigen rather than its destruction.

(2) Stability The intensity of nucleolar staining after various

fixation procedures in one experiment is shown in Table 4/2. In most other experiments the nucleolar antigen has shown similar stability to fixation but the results have not been completely reproducible as the antigen has been destroyed occasionally by the mildest treatment (absolute acetone for 10 min. at room temperature). The "nucleolar" antigen is somewhat more labile to chemical fixation than the "speckled" antigen but like the latter, it is apparently destroyed perferentially by protein denaturing fixatives, suggesting that there may be a protein moiety in the nucleolar antigen.

As absolute acetone usually had little effect on the antigen at room temperature, this has been the fixative of choice for destruction of cell membrane selective permeability before staining the intact cells in tissue cultures. The nucleolar antigen could not be demonstrated in sections of guinea pig liver and lymph node prepared by the freeze substitution method (Balfour, 1961) but this is hardly surprising in view of the lability of this antigen in absolute ethanol at room temperature.

(3) Enzymic digestion The results of staining by MJI. or SCR. after various enzyme treatments are summarised in Table 4/3 which shows that RNase and proteases prevented staining whereas DNase did not damage the "nucleolar" antigen. These findings suggest that the antigen contains RNA and protein components but subsequent experiments show that this simple direct deduction is untenable (see p. 68).

(4) The staining properties of isolated nuclei and nuclear fragments

Intact nuclei were isolated and extracted as described in Chapter 3 (p. 55) and they were stained by MJI. and SCR. Table 4/4 shows that the nucleolar antigen was removed by extraction with 0.15M-NaCl, confirming the observations on frozen sections.

In a series of experiments in which I received considerable technical assistance from Miss S. Waddington (Biochemistry Department, The University, Glasgow), attempts were made to disintegrate isolated rat liver cell nuclei.

(a) By mechanical trauma in a Potter-type tephlon-cylinder tissue homogeniser or

(b) By treatment at 20 Kc./sec. in 0.25M sucrose for periods up to 10 min. at 0°C to 5°C in a Mullard ultrasonic disintegrator in an atmosphere of hydrogen.

Fractions were then prepared by differential centrifugation in sucrose medium and stained by MJI. Mechanical disintegration was unsatisfactory as the nuclei either survived intact or were broken into small fragments in which the nucleolar antigen could not be identified. Ultrasonic treatment was somewhat more promising since, in one experiment, small spherical bodies could be stained in the deposit obtained after centrifugation at 700 r.p.m. for 5 min. These bodies were similar in size and shape to rat liver cell nucleoli and were embedded in unstained fragments of nuclear material (fig.4/1). The conditions for ultrasonic disintegration of nuclei must be very

critical as subsequent experiments (following closely the conditions employed in the partially successful pilot run) have always been unsuccessful and yielded either undamaged nuclei or amorphous debris.

(C) Fluorescent Spot Test

Attempts were made to stain dried films of the various tissue constituents by MUI. or SCR., but in no case was specific staining obtained.

(D) Staining with Antinucleolar Sera after Absorption with Various Tissue Constituents

(D) Staining with Antinucleolar Sera after Absorption with Various

Attempts were made to absorb the nucleolus-staining capacity of MUI. and SCR. by absorption for 1 hr at 37°C with the purified tissue components listed on p. 40 . Treatment with rat liver cell nuclei (isolated in 0.25M sucrose) absorbed the antinucleolar antibody but the other tissue products did not absorb the antibody. Attempts at absorption with many preparations of ribonucleic acid and ribonucleoprotein (supplied by Dr H. Munro) and 0.1M phosphate buffer and 1.0M-NaCl extracts of isolated nuclei (supplied by Miss S. Waddington) were also unsuccessful. It has not been possible to test the "nucleolus-rich" fraction of disrupted nuclei as this fraction has not yet been obtained in sufficient quantity. The significance of these findings will be discussed in detail.

(E) Annulment Experiments

These were performed using a technique similar to that described on p. 41 , except that acetone-fixed cryostat sections of rat liver

were used because of the solubility of the nucleolar antigen in saline. Pre-treatment by any of the antinucleolar sera prevented staining by directly conjugated MUI., whereas none of 6 "speckled" sera (Tra., Flu., Ral., McD., McLau. and Nis.) nor 6 "homogeneous" sera (Dun., Mot., McL., Wex., Bry. and Pea.) prevented staining by directly conjugated MUI.(Beck et al., 1962).

DISCUSSION

Chemistry of the nucleolar antigen

The investigations described in this chapter showed that the nucleolar antigen was fairly labile and widely distributed in the nucleoli of vertebrate cell nuclei. It was removed by treatment with 0.15M- or 1.0M-NaCl but it has not been possible to determine whether this was due to simple extraction or chemical degradation. It was also removed by the action of either RNase or proteolytic enzymes. The antinucleolar antibody was removed from serum by absorption by isolated cell nuclei but not by any of the extracts of nuclei which were tested. It has not been possible to identify the nucleolar antigen.

The action of RNase and proteolytic enzymes suggested that it might be nucleolar ribonucleoprotein or a constituent of this molecule, but it could equally well be some substance which was normally held in the nucleolus in association with the ribonucleoprotein. The failure of all the absorption experiments is puzzling.

It must, however, be remembered that an antigen in a tissue extract could be readily degraded by dissociation of complex molecules, by the action of cellular enzymes released during homogenisation of tissue or by spontaneous decay of a molecule (messenger RNA has a very short half life). Furthermore, the nucleolar antigen might well be present only in trace amounts in nucleoli, so that it would be present in such small quantities in extracts that it could not be detected.

There can be little doubt that the nuclear and nucleolar RNA and ribonucleoprotein preparations obtained from the Biochemistry Department of the University of Glasgow were highly purified and biochemically intact (with the reservation that messenger RNA would probably have decayed spontaneously during the period which elapsed before testing). As these preparations did not absorb the antinucleolar antibody, it seems highly improbable that either RNA or ribonucleoprotein could be the nucleolar antigen. Furthermore, the nucleolar antigen did not decay rapidly in sections or frozen tissue as it could be detected up to 3 weeks after removal of the tissue from the animal; therefore, it is highly unlikely that the antigen is messenger RNA in view of the short half life of this substance.

Since neither ribonucleic acid nor ribonucleoprotein appeared to be the nucleolar antigen, it was probable that the antigen was a substance (possibly a protein) closely associated with nucleolar

ribonucleoprotein which could be released by the action of RNase or proteolytic enzymes. Further investigations are being undertaken in an attempt to determine whether the nucleolar antigen is a protein, lipid or carbohydrate as these substances have been identified by histochemical techniques in nucleoli (see Sirlin, 1962).

Other investigations on antinuclear antibodies by the fluorescent antibody technique

Since my first description of antinucleolar antibody (Beck, 1961b), the presence of these antibodies in the serum of patients with "connective tissue" diseases has been confirmed by Bardawil et al. (1962), Fennel et al. (1962) and White and Marshall (1962). Bardawil et al. (1962) have shown that the staining can be augmented by pretreatment of the section with RNase, confirming my findings but they claim that the serum factor can be absorbed by yeast RNA, although I have been unable to repeat this. The other investigators have not investigated the chemical nature of the nucleolar antigen. The antinuclear antibody has not been demonstrated by any technique other than immunofluorescence.

Maisel (1962) has demonstrated recently non-immunological attachment of α globulin from normal serum to nucleoli but he has not identified the chemical nature of the reacting constituent in nucleoli. In view of the specificity controls (Chapter 1) and demonstration of the γ globulin nature of the antinucleolar antibodies (Chapter 6) studied in this investigation, it is certain that

the phenomena described in this Chapter do not correspond to the non-immunological serum factors studied by Maisel (1962).

SUMMARY

The nucleolar antigen has not been identified but the experimental evidence suggests that it is a substance closely associated with ribonucleoprotein. It is certainly neither RNA nor ribonucleoprotein.

CHAPTER 5

THE "MEMBRANOUS" ANTIGEN

The membranous pattern of nuclear staining was the least common staining pattern encountered in the fluorescent antibody test for antinuclear antibodies (Table 8/38). It was found with only 3 patients' sera (Nob., McDou. and Tan.) and in all these sera a precipitating antibody to DNA had been demonstrated previously (Anderson et al., 1962). The experiments recorded below show that the serum factor producing the "membranous" pattern of nuclear staining and the precipitating antibody to DNA are almost certainly identical.

(A) Organ and Species Distribution of the "Membranous" Antigen

The findings are summarised in Table 5/1. Rather fewer varieties of cell nuclei have been stained by the "membranous" sera than by antinuclear sera of the other types, because the "membranous" pattern was recognised at a later stage in the investigation when many tissues were no longer available. The "membranous" antinuclear sera stained the nuclei of all cells except spermatozoa. Unfortunately, tissue culture cells with cytoplasmic DNA virus inclusion bodies have not been available for study by the "membranous" antinuclear sera.

(B) Reaction of "Membranous" Antinuclear Sera with Nuclei modified by Histochemical Procedures

The altered nuclei were stained subsequently by the 3 "membranous"

antinuclear sera (Nob., McDou. and Tan.).

(1) Extraction After pretreatment with distilled water or 1.0M-NaCl the appearances were similar to those seen on staining with "homogeneous" antinuclear sera. However, after treatment with 0.15M-NaCl, the nuclei showed marked swelling similar to the "shaggy" appearance described by Casals et al., 1963. This difference in behaviour between the "homogeneous" and "membranous" antigens on extraction with 0.15M-NaCl was probably important as DNA was slowly soluble in 0.15M-NaCl whereas nucleohistone was insoluble.

(2) Stability The intensity of "membranous" staining after various fixation procedures is shown in Table 5/2. It was noteworthy that the "membranous" antigen was not damaged by protein denaturants nor by osmic acid which oxidises unsaturated lipids. Furthermore, this antigen survived for several weeks in sections or smears standing at room temperature and almost indefinitely when the preparations were stored at $-18^{\circ}C$ in a dessicator.

(3) Enzymic digestion The results of staining by the "membranous" antinuclear antibody after various enzyme treatments are shown in Table 5/3. "Membranous" staining was prevented by pretreatment with DNase but not by the other enzymes tested.

(C) Staining with "membranous" antinuclear sera after absorption with various tissue components

A series of experiments was undertaken to determine whether isolated cellular organelles and purified tissue extracts were capable of

absorbing the "membranous" antinuclear antibody ~~for~~ from sera ~~for~~ of patients (Nob., McDou. and Tan.) The antibody was successfully absorbed by whole liver cell nuclei, nucleohistone and DNA. Absorption with the other extracts did not remove the capacity of the sera to produce "membranous" staining of cell nuclei.

DISCUSSION ON Identification of "Membranous" Antigen

Identification of "Membranous" Antigen

The problem of identification of the antigen responsible for "membranous" staining was somewhat simpler than in other systems because all sera giving this pattern of staining had been shown previously to contain anti-DNA antibody with an Ouchterlony precipitin system (Anderson et al., 1962).

The experimental evidence presented above can be summarised thus:-

1. The "membranous" antigen was present in the nuclei of all cells examined except those of spermatozoa.

2. The solubility and stability of the "membranous" antigen were closely similar to those of free DNA.

3. The susceptibility of the "membranous" antigen to enzymatic digestion was similar to that of free DNA.

4. The "membranous" antinuclear antibody could be absorbed from patients' sera by whole nuclei, nucleohistone and free DNA but not by any other tissue component.

The characteristics of the "membranous" antigen were closely similar to those of DNA; the only discrepancy was the consistent

failure of "membranous" antinuclear sera to stain spermatozoal nuclei. Investigation of this problem is continuing and three possible explanations are being considered.

(i) The cell membrane of spermatozoa is not permeable to antibody.

(ii) DNA is so densely packed in the spermatozoal nucleus that the antibody cannot penetrate.

(iii) The antigenic determinant groups of the DNA are blocked by the combined protamine.

It seemed highly probable that the failure of staining of spermatozoal nuclei by anti-DNA sera would be attributed untimately to a failure of penetration of the antibody rather than to blocking of the antigenicity of DNA by combination with protamine since Lachmann (1961b) has claimed that spermatozoal nuclei can be used as a substrate for passive L. E. cell tests with "connective tissue" disease sera. It was, however, possible that Lachmann's findings had resulted from reaction of anti-nucleohistone with nucleohistone derived from the nuclei of somatic cells contaminating his preparations of spermatozoa.

It was unfortunate that DNA virus cytoplasmic inclusion bodies have not been available since the "membranous" system has been recognised because demonstration of a staining reaction by the "membranous" antinuclear antibody on these inclusion bodies would have been strong evidence that the "membranous" system depends upon the presence of anti-DNA in the serum. Rapp and Friend (1962) have

shown previously that certain "connective tissue disease" sera stain cytoplasmic particles of a DNA virus by the immunofluorescent technique, but they did not characterise the antibody responsible for this staining reaction.

No attempt has been made to demonstrate specific blocking of the staining reaction produced by a directly conjugated "membranous" antinuclear serum by other "membranous" sera, because these sera were in short supply. It seems highly probable, in view of my experience with the other systems of antinuclear antibodies, that all the "membranous" antinuclear sera react with the same antigen.

I think that the "membranous" pattern of nuclear staining is almost certainly produced by anti-DNA but this cannot be considered as adequately proved until the failure of staining of spermatozoal nuclei can be explained. This is not a revolutionary concept as autoantibodies to DNA have been demonstrated previously by all the conventional immunological techniques (Table 5/4).

There is, furthermore, evidence that the double helix structure is not essential for antigenicity in DNA as Barbu et al. (1960) and Stollar and Levine (1961) have shown that heat denatured DNA is still active.

Anderson et al. (1962) have demonstrated that, in one case, the DNA precipitin is an autoantibody since it reacts with DNA prepared from the patient's own spleen. In this same patient (Tan.) I have shown that her serum produces "membranous" nuclear staining in

sections of her liver.

Despite the widespread recognition of anti-DNA autoantibodies by conventional immmnological techniques, there have been reports from only one research group (Casals et al., 1962, 1963) claiming the recognition of this antibody by the fluorescent antibody technique; in this, they described marginal nuclear staining by sera which had been shown to contain anti-DNA in a complement fixation test and they were able to absorb the staining capacity of these sera by absorption with DNA. These results are similar to my findings described above. Gökcen (1962) has claimed to have recognised antibodies to two different types of DNA in leucocytes by human antinuclear sera but his experiments were poorly controlled and his results could be explained equally well by the presence of antinucleohistone and "speckled" antinuclear antibody in his sera (see Chapter 11).

No attempt has been made in this investigation to determine the precise antigenic determinants on the DNA molecule which react with anti-DNA ("membranous") antinuclear sera. It seems highly probable, however, that they will prove similar to the anti-DNA autoantibodies described by Stollar et al. (1962) and Stollar and Levine (1963) which appear to react with either penta-nucleotides or purine residues respectively.

The relationship of the "Membranous" Antigen to the structure of the nucleus

The observation that sera containing anti-DNA and anti-

nucleohistone stained nuclei with different patterns was quite unexpected. There was no doubt that sera containing "membranous" antinuclear antibody (anti-DNA) stained individual nuclei at different intensities (the cut nuclei showing more intense fluorescence), that the staining was much brighter at the margins of the nuclei while the perinucleolar heterochromatin was prominent.

Interpretation of these findings was difficult. While it is possible that the brighter staining of cut nuclei and the ring of intense staining at the edge of the nuclei might result from certain "artefacts" (e.g. easier access of precipitating anti-DNA or preferential exposure to cytoplasmic or serum enzymes) the prominence of the heterochromatin cannot be explained on this hypothesis. This must raise the question of a chemical difference in the heterochromatin (e.g. DNA more loosely complexed, present in higher concentration or attached to non-histone protein) and this would be supported by the classical observations that the staining reaction of the heterochromatic with conventional dyes are different from the rest of the chromatin.

It is possible therefore that the "membranous" pattern of nuclear staining may be explained ultimately by differences in antigen location coupled with differences in accessibility to antibody.

SUMMARY

The "membranous" pattern of nuclear staining has been produced

only by sera containing a precipitating autoantibody to DNA. The evidence presented in this Chapter shows that the two factors are almost certainly identical.

CHAPTER 6

THE PROPERTIES OF ANTINUCLEAR ANTIBODIES

There has been some reluctance to accept as antibodies the serum factors which react with cell nuclei. This has been demonstrated by certain authors who refer to them as the "antinuclear factors" or use some other non-commital term (Holborow et al., 1957; Alexander et al., 1960; Holborow, 1960; Pollak et al., 1960; Couchman et al., 1961; Fennell et al., 1962; Weir and Holborow, 1962). There is, however, a growing opinion that the 7.0S or 19.0S immune globulin antinuclear factors must be regarded as antibodies as they show considerable specificity of reaction (Holman, 1960; Doniach and Roitt, 1962).

The reluctance to accept the immunological nature of the antinuclear factors was probably partly by caution to avoid making unsupportable claims and partly by difficulty in correlating the lack of species specificity with the accepted immunological criteria of specificity. It is now, however, becoming realised that although not species specific, the reaction of certain antinuclear antibodies may show a high degree of chemical specificity as Levine and his associates have shown that certain lupus erythematosus sera containing anti-DNA can be effectively inhibited by pentanucleotides (Levine, 1962) and that one serum can be inhibited by mononucleotides and free bases (Stollar and Levine, 1962).

This chapter describes experiments I have undertaken to determine the physico-chemical nature of the serum factors which react with cell nuclei.

MATERIALS AND METHODS

1. Ultracentrifugation of Patients' Sera.

Separation of serum proteins was undertaken with the assistance of Dr P. A. Charlwood (National Institute for Medical Research, London) by the radioactive labelled marker technique devised by Charlwood, (1963). For this, highly purified 7.0S and 19.0S fractions of normal human serum was labelled with a radioactive isotope (usually ^{131}I, but in a double marker test ^{125}I was also employed, by the method described by McFarlane in 1958. A trace amount of the marker protein was then mixed with the patient's serum which was then layered on top of a sucrose density gradient (10% to 30% sucrose) in a plastic tube. This was then centrifuged in a Spinco model E centrifuge at 25,000 r.p.m. in a rotor 40 head for 18 hr at room temperature. Thereafter the base of the tube was punctured with a hollow needle and fractions withdrawn. The fractions were counted in a scintillation counter and tested for the presence of antinuclear activity on rat liver by the usual method.

2. Chromatographic Separation of γ Globulin from Patients' Sera

The γ globulin fractions of sera containing antinuclear activity were prepared by elution with 0.02M phosphate buffer (pH 6.3) (Fraction A) from the charged DEAE Sephadex columns (see Appendix

1/1). Further serial stepwise elutions were made with sodium chloride solutions whose pH was controlled with 0.02M phosphate buffer pH 7.0 and the following fractions were obtained -

Fraction B (0.075M-NaCl) mainly β globulin

Fraction C (0.15M-NaCl) albumin, α_1, and α_2 globulin.

Fraction D (0.4M-NaCl) α_2 and β globulin.

All fractions were concentrated by pressure dialysis back to the starting serum volume and tested for antinuclear activity by the usual method.

RESULTS

1. Ultracentrifugation

The results of the experiments with 9 sera containing high titre antinuclear activity are shown in Table 6/1 and figs. 6/1, 6/2 and 6/3. These sera were obtained from 4 cases of systemic lupus erythematosus, 3 cases of Sjögren's syndrome and 2 cases of progressive systemic sclerosis. Charlwood (1963) has shown that the radioactive iodine label does not become detached from the marker protein during ultracentrifugation, so that the number of counts indicated the relative quantity of marker protein in each fraction.

Antinuclear activity was greatest in the fractions with the highest counts of labelled marker 7.0S protein. As the radioactive iodine labelled 7.0S marker protein did not react with cell nuclei when tested at a much higher concentration than that found in any fraction, it was obvious that the antinuclear activity in each

patient's serum had the same ultracentrifugal characteristics as the 7.0S marker protein. This demonstrated that the antinuclear activity in the 9 sera tested had a sedimentation coefficient of 7.0S. In one case (McDon.) a double marker tracing technique was used with normal human 7.0S globulin labelled with ^{131}I and normal human 19.0S globulin labelled with ^{125}I. Fig. 6/3 shows that the antinuclear activity behaved in the same way as the 7.0S marker protein whereas the 19.0S macroglobulin was quite distinct and was found in lower fractions. Several other sera were subsequently shown to be resistant to treatment with 0.2M mercapto-ethanol (Chapter 9) using a modification of the technique described by Weir and Holborow (1962). This was further evidence that the antinuclear factor is not a 19.0S macroglobulin in these sera.

One serum (McDia.) from a patient with atypical complicated rheumatoid arthritis behaved quite differently on ultracentrifugation (Table 6/2) and fig. 6/4. The antinuclear activity and 7.0S marker protein were not associated and the distribution of the antinuclear activity corresponded to the expected position of 19.0S macroglobulin in the lower tubes. Unfortunately, it has not been possible to repeat the test with the double marker system as the supply of serum was exhausted and the patient had died.

2. DEAE Sephadex Chromatography

The results of tests for antinuclear activity in the separated fractions of 4 patients' sera are shown in Table 6/3. In each case,

antinuclear activity was present in Fraction A only. All the (7S) globulin comes off the column in this fraction which contains very little protein of other types.

3. Electrophoresis

Two sera (Tra. and Dun.) were electrophoresed on filter paper and fractions extracted as described previously (Beck, 1961a). In each case, antinuclear activity was restricted to the γ band.

DISCUSSION

Physico-chemical Nature of Serum Factor reacting with Cell Nuclei

It has been shown in Chapter 1 that these factors have the antigenicity of human γ globulin. The experiments reported in this chapter showed that 9 of the 10 sera tested behaved on ultracentrifugation like 7.0S globulin. The antinuclear factor in the other serum (McDia.) sedimented more rapidly; the experimental results suggested that this serum factor was a 19.0S macroglobulin, but this could not be proved conclusively. DEAE Sephadex chromatography and electrophoresis of some of the sera in the first group showed that the antinuclear factor behaved like a γ globulin. Therefore, as the antinuclear factors have similar antigenicity, sedimentation coefficient and molecular charge to the immune globulins it seemed reasonable to conclude that the antinuclear factors were in fact immune globulins.

Very little attention has been paid previously to the nature of the antinuclear factor detected by the fluorescent antibody technique,

although most workers have shown that it has the antigenicity of human γ globulin. Goodman et al. (1960) have shown that in some sera the factor was a 7.0S globulin whereas in other sera it was present in both 7.0S and 19.0S globulin fractions. Weir and Holborow (1962) have investigated sera by mercapto-ethanol treatment to inactivate 19.0S macroglobulins and shown that the antinuclear antibodies in systemic lupus erythematosus sera was resistant whereas this treatment partially or completely destroyed the antinuclear antibody in some rheumatoid arthritis, liver disease and thyroiditis sera. Recently, Baum and Ziff (1962) carried out ultracentrifugation studies and showed that antinuclear antibody was predominantly 7.0S in systemic lupus erythematosus sera and predominantly 19.0S in rheumatoid arthritis sera. The high incidence of 7.0S antinuclear antibodies encountered in my sera is easily explained as systemic lupus erythematosus, progressive systemic sclerosis and Sjögren's syndrome sera were selected because they contained high titre antinuclear antibodies.

In contrast, the nature of the L. E. cell factor has been investigated thoroughly and it has been established with zone and Tiselius method electrophoresis that it was a γ globulin (Haserick et al., 1950; Holman and Kunkel, 1957; Carlson and Möllenberg, 1958). This has been confirmed by CM cellulose chromatography by Fallet et al. (1959) and Willkens et al. (1958). Holman and Deicher (1959) and Larson et al. (1959) have shown that the L. E. cell factor has

a sedimentation coefficient of 7.0S.

There has, however, been little investigation of the nature of the other antinuclear antibodies detected by more conventional techniques. Seligmann (1958) has shown that anti-DNA is antigenically identical with human γ globulin and Deicher et al., (1959, 1960) and Anderson et al. (1962) have demonstrated that this factor behaves like γ globulin in electrophoresis. Anti-nucleohistone has been investigated by ultracentrifugation and DEAE cellulose chromatography by Goodman et al. (1960) who have shown that in some sera this factor is found in the 7.0S fraction but in others it is divided between the 7.0S and 19.0S fractions. Other antinuclear antibodies to histone and phosphate buffer extract of isolated nuclei have been demonstrated by Holman et al. (1959) but so far no attempt has been made to establish the nature of the serum factor involved.

Antibody nature of antinuclear factor

The experiments described above have shown that the antinuclear serum factors, which have been detected by the fluorescent antibody technique are immune globulins. In Chapters 2 - 5, it has been shown that the 4 serum factors react specifically with 4 distinct intranuclear antigens. The only possible objection to acceptance of these serum factors as antibodies would be the lack of species specificity in the antigens with which they react. So far as the antigenicity of DNA is concerned, this objection is unlikely to be valid since recent work on the immunochemistry of this antigen using

a complement fixation technique has shown that in some cases the antigenic determinant is a pentanucleotide (Stollar et al., 1962a) and in one case a mononucleotide (Stollar and Levine, 1962). It is certain that similar sequences of 5 or fewer nucleotides will be common to all samples of DNA, no matter their source. The antigenic determinants with which the other antinuclear antibodies react have not yet been determined. However, as the parent molecules (such as nucleohistone) are fundamental biochemical units of nuclear structure, it would hardly be surprising that their antigenic determinants could occur in a wide range of species, in view of the experience with DNA.

SUMMARY

The serum factors responsible for nuclear staining in the fluorescent antibody technique must be considered antibodies because they react specifically with the corresponding antigens and have the physico-chemical properties of immune globulins (7.0S γ globulins or 19.0S macroglobulins).

PART III

(Chapters 7, 8 and 9)

CLINICAL STUDIES

CHAPTER 7

THE INCIDENCE OF ANTINUCLEAR ANTIBODIES IN HOSPITAL PATIENTS IN WHOM "AUTOIMMUNE" DISEASE WAS NOT SUSPECTED

It has been established in many previous investigations (e.g. Weir et al., 1961) that antinuclear antibodies were present in the serum of many "connective tissue disease" patients, but that these antibodies were rare in apparently normal individuals. Furthermore, these antibodies were usually strong in the patients but weak in the normal subjects. It was therefore important to determine the incidence and titres of antinuclear antibodies detected by my fluorescent antibody technique in a control series before using this technique to study the "connective tissue diseases".

MATERIALS AND METHODS

The problems involved in selection of a population of normal subjects have been emphasised in a Lancet Annotation (1960). Blood donor sera were readily available but these were not surveyed because it would have been very difficult to trace and examine any subject whose serum produced nuclear staining. Instead, consecutive sera submitted for blood grouping at the Haematology Department (Western Infirmary, Glasgow) were tested as most of these sera came from surgical or gynaecological patients whose case records were available. I realised that this group is not a normal population as I had shown previously that a similar group had a significantly higher incidence

of "antiantibody" than blood donors (Beck, 1961a), but putative "autoimmune" disease was not diagnosed in any of them so that this was considered the best available control group to compare with patients with diseases in which autoimmunity has been incriminated as a pathogenetic mechanism.

Five hundred sera were tested. These were as far as possible consecutive blood grouping sera. The only selection was the omission of duplicate specimens, those where the volume of serum was inadequate for testing and those from patients with putative autoimmune disease.

All sera were tested undiluted and diluted 1/4 in barbitone buffered saline (pH 7.2) with further titration at fourfold dilutions if necessary. Cryostat sections of rat liver were stained by the method described in Chapter 1. The staining times were strictly standardised at 30 min. for each stage. A single batch of fluorescein-conjugated rabbit anti-human-γ-globulin was used throughout and the specificity of this reagent was controlled by demonstrating specific absorption by highly purified human γ globulin and also by "blocking" with unconjugated anti-human-γ-globulin using the methods described in Chapter 1.

RESULTS

Autofluorescence, although a feature of cytoplasm, did not occur in the nucleus with the dark ground optical system used in this investigation. Unstained nuclei were seen as dark areas in

the centre of the hepatic cells and it was possible to get a good end point in titration of antinuclear activity of sera. The patterns of nuclear staining were classified as described in Chapter 1 except with sera giving faint staining only when undiluted as the bright cytoplasmic staining produced by undiluted serum obscured the nuclear staining pattern.

The results of this survey are shown in Table 7/1 where the staining patterns and titres are compared with the age and sex of the patients. The ratio of the incidence of "homogeneous" and "speckled" staining at various titres is shown in this table but there was no relationship between staining patterns and titres ($\chi^2 = 3.565$, $n = 4$: $P = 0.3$).

The incidence of nuclear staining at any serum dilution is compared with age in females and males in Tables 7/2 and 7/3 and figs. 7/1 and 7/2 respectively while they are graphed as percentages in figs. 7/3 and 7/4 where the limits of two standard deviations are indicated for each age group. Comparison of the incidence of nuclear staining with age in men shows that it is highly probable that the observed differences could occur by chance. In women, however, the incidence of nuclear staining varies greatly at different ages and it has been shown by the χ^2 test that the observed incidences could occur by chance less than once every 100 times when sera were diluted 1/4 with saline, but the differences are much less significant ($P < 0.1$) when undiluted serum was used. Fig. 7/3 shows

that the incidence of nuclear staining is significantly lowered in women in the age group 40 - 60 years ($P<0.05$). There is a tendency to increased incidence at older age groups and this is observed consistently in women in the 8th decade. The high incidence in females less than 30 years is not significant as only small numbers were studied in each of these decades (Table 7/2).

DISCUSSION

In any quantitative investigation with the fluorescent antibody technique, it is important that the immunological techniques are carefully standardised and that the optical apparatus remains unchanged throughout the experiment, as differences in the light source, optical system or filters can modify the sensitivity of the observations (Goldman, 1960; Goldman and Carver, 1961). In this survey, the serum volumes and staining times were strictly standardised and one batch of fluorescein-conjugated anti-human-γ globulin was used throughout. The sensitivity of the ultraviolet microscope cannot have altered appreciably as the observations were made during the first 50 hr use of a Mazda ME/D discharge lamp whose light emission does not deteriorate significantly until 150 hr.

The nature of weak nuclear staining reactions

It is difficult to determine conclusively whether weak nuclear staining is produced by antinuclear antibodies, as such reactions are not suitable for absorption studies to establish specificity. However, the serum factors must be γ globulin in view of the strict

specificity of the fluorescent anti-human-γ-globulin reagent employed, so that non-immunological reactions of α globulin (Maisel, 1962) or partly denatured human serum albumin (Beck, 1962b) with nuclear constituents cannot be involved.

In view of my previous observation that human serum albumin may be altered spontaneously or by mild oxidation so that it can adhere to cell nuclei (Beck, 1962b) it is important to consider whether the staining reactions observed in this survey might be the result of physical or chemical alteration of γ globulin after the patient was bled. Such alteration could be caused by chemical contamination (e.g. detergent) from glassware or by bacterial growth in the serum during storage. It is, however, highly improbable that such non-immunological artefacts could be responsible for weak nuclear staining activity in view of the raised incidence of this phenomenon in elderly females. The results must reflect the activity of a γ globulin which was present in the patient's blood and it is highly probable that such γ globulin is an antibody. Laffin et al. (1963) have also detected antinuclear globulin in some "normal" sera.

The significance of variations in incidence in different age groups

The prevalence of antinuclear antibody was similar in the males in all age groups. In females, however, the incidence was definitely lowered in the 5th and 6th decades and raised in the 8th decade, while the increased incidence in women in the 7th decade could only be shown

to be statistically significant at certain levels of sensitivity of the test. Although there was a high incidence in women less than 30 years, this was not statistically significant because of the small numbers studied. Unfortunately, it was not possible to continue the survey after analysis of these results to enlarge the groups of young women as the batch of fluorescein-conjugated anti-human-γ-globulin was exhausted.

It is interesting to compare these findings with surveys of antithyroid antibodies in patients without clinical evidence of thyroid disease. Goudie et al. (1959) found an increased incidence of complement fixing antibodies to thyroid extract in women aged 50 - 80 yr, whilst Hackett et al. (1960) and Hill (1961) showed an increased incidence of antithyroglobulin in women aged 30 - 80 and 40 - 70 yr respectively. Hackett et al. (1960) showed that the increased incidence persists in women over the age of 80 years but in the other two series there is a fall in incidence in the oldest groups of women studied. Since titres of antithyroglobulin tend to fall in old age, Anderson et al. (1961a) have suggested that Hackett et al. (1960b) used a more sensitive test and so were able to detect the raised incidence in women over 70 yr.

It has been suggested that women over 40 yr are more liable to produce antithyroid antibodies because of immunisation by discharge of tissue antigens resulting from changes in thyroid metabolism at the menopause, but it must be remembered that thyroid antigens are

also released to the blood during pregnancy and the puerperium (Hjort and Pedersen, 1962). The increased incidence of antinuclear antibodies in older women described in this Chapter suggested that there may be a tendency to breakdown of immunological tolerance in such women. If this were so, it would not be surprising that release of thyroid antigen would result in immunisation in post-menopausal women but less commonly in younger women. This is analogous to the finding in the NZB/BL strain of mice where auto-immune haemolytic anaemia frequently develop in old age and Holmes et al. (1961) have suggested that the loss of immunological tolerance is the result of the emergence of forbidden clones in old age. It was interesting, therefore, that Aho et al. (1961) found that the mean age of normal subjects with rheumatoid factor was considerably higher than that of the normal subjects without serological abnormality during a random sampling study of the population of a small town; unfortunately, the sexes were not separated in this study.

Burch (1963a) has proposed that autoimmunity results from somatic mutations of immunologically competent cells and he has estimated the number of mutations involved in rheumatoid arthritis (Burch, 1963a) and in discoid and systemic lupus erythematosus, systemic sclerosis and Hashimoto's thyroiditis (Burch and Rowell, 1963) from the estimated age of onset of the disease. The mathematical theory on which this is based (Burch, 1963b and c) has been disputed by Maynard Smith and Maynard Smith (1963a and b). Although I am not qualified

to judge the mathematical arguments, it appeared reasonable to plot the data in Tables 7/2 and 7/3 on a double logarithmic scale (fig. 7/5) in the way Burch studied rheumatoid arthritis. This figure showed that the age-specific incidence was not linear. Since there was no evidence that antinuclear antibodies were capable of causing tissue damage (Chapter 9), the age-specific incidence should have approximated closely to the age-specific incidence rate. The observed data was not linear, therefore it must be concluded either that the formation of antinuclear antibodies was not induced by somatic mutations or that Burch's hypothesis was not valid.

Definition of titres of pathological significance

Before using a laboratory technique to study disease, it is essential to evaluate and, if necessary, adjust its sensitivity so that it will give optimal differentiation between normal and diseased subjects. Reconsideration of Tables 7/1, 7/2 and 7/3 made it obvious that it would be unsatisfactory to use serum neat or diluted 1:4 with saline in my staining technique as a high proportion of "false positives" would be found in control patients. A serum dilution of 1:16 appeared the best compromise between sensitivity and pathological significance as there were only 4% "false positives" at this level. This dilution was therefore used for the screening test on pathological sera in the clinical part of this investigation.

It is realised that the data presented in this Chapter cannot be used to control clinical investigations as the sensitivity of the

test depends upon optical factors such as the age of the discharge lamp. This survey has, however, emphasised that any control series must be matched for age and sex with the group of patients who are being investigated for antinuclear antibodies.

SUMMARY

Five hundred blood grouping sera were tested for their capacity to produce nuclear staining in the fluorescent antibody test for antinuclear antibody. 46.2% of the sera were positive when tested undiluted, 26.6% at 1/4, 4% at 1/16 and 0.2% at 1/64 dilution. The incidence was markedly raised in women over 60 years of age but there was no age variation in men. On the basis of this series, it was decided that sera would be used at 1/16 dilution in the screening test for antinuclear antibodies.

CHAPTER 8

THE INCIDENCE OF ANTINUCLEAR ANTIBODIES IN HUMAN DISEASES

It is now well established that antinuclear antibodies and other autoantibodies are a prominent feature in systemic lupus erythematosus and that these phenomena are also encountered in the other "connective tissue diseases". This chapter describes the results which I have obtained by the fluorescent antibody test for antinuclear antibodies in patients with "connective tissue diseases" and certain other diseases in which autoimmunity was a prominent feature. When this investigation was started the fluorescent antibody test for antinuclear antibodies was not fully established as a diagnostic test. In order to assess its clinical value, it was important to compare the results of the fluorescent antinuclear antibody test with those obtained by other immunological and biochemical techniques in common use. Ideally, I would have performed these other tests personally but this was not possible in the time available and so I have compared my results with those obtained in other immunological and biochemical laboratories. These results will be useful for comparison with my fluorescent antibody test for antinuclear antibodies only when a series of tests have been performed in the same laboratory on blood samples withdrawn at the same time as the specimens tested for antinuclear antibodies. Because of these restrictions, it has not been possible to make comparisons with all

the better known immunopathological investigations in each of the diseases which was studied. Table 3/1 indicates the tests which will be considered in relation to the principal diseases discussed in this Chapter.

It must be realised that the results which are presented are primarily those of a laboratory investigation on antinuclear antibodies and that I have been largely dependent on my clinical colleagues for the selection of patients. When, however, there was doubt about the diagnosis, I have taken final responsibility for the classification of the patient for this thesis, usually after consultation with the clinician but sometimes after survey of the case records.

The results of the fluorescent antibody tests for antinuclear antibodies have given further evidence for the classification of the "connective tissue diseases" as a group of closely related diseases but they pose questions on the relation of this group to the so-called "organ-specific autoimmune diseases".

MATERIALS AND METHODS

It will be convenient to describe the laboratory methods in this introduction as certain tests have been applied to patients with different clinical syndromes; the criteria used for the selection of patients will be discussed separately under each disease.

Antinuclear antibodies were detected by the fluorescent antibody method described on page 22. All sera were screened at 1/16 dilution in

buffered saline and if positive, titrated at fourfold dilutions. The pattern of nuclear staining was noted in all positive preparations. The great majority of the Glasgow sera were ~~treated~~ tested without prior knowledge of the diagnosis. The Leeds and Washington sera were identified by code numbers and each batch included sera from normal individuals and from patients with various diseases, in which there was no evidence of autoimmunity.

L.E. cell tests were reported by the local laboratories of the hospitals where the patients were being treated. In most tests, the method of Zimmer and Hargraves (1952) was used but for the investigations of the Washington Sjögren's syndrome patients, the technique developed by Zinkham and Conley (1956) was chosen.

Precipitin tests for tissue autoantibodies were detected by the Ouchterlony precipitin system described by Anderson et al. (1961b, 1962). All sera were tested against an extract of human thyroid and, if positive, against extracts of other human organs.

Antibodies to parietal cells in human gastric mucosa Cryostat sections of fresh human gastric mucosa (usually blood group O) were treated with serum diluted 1:5 with barbitone-buffered saline and stained by fluorescent anti-human-γ-globulin. Staining and washing times were standardised at 30 min. and 10 min. respectively. Sera were considered positive if they produced distinct staining of the parietal cells.

Rheumatoid factor tests were reported by the laboratories of the

hospitals where the patients were being treated. Different experimental techniques have been used in each centre, namely

(a) Hyland "R.A." latex test: Western Infirmary, Glasgow.

(b) Bentonite flocculation test (Bozicevich et al., 1958): Sjögren's syndrome patients, National Institutes of Health, Washington.

(c) Sensitized sheep cell agglutination test (Ziff et al., 1956): Sjögren's syndrome patients, National Institutes of Health, Washington.

(d) Classical Rose-Waaler test: "connective tissue disease" patients, General Infirmary, Leeds.

Antithyroglobulin Tanned red cell tests for the detection of this antibody in the sera of the Glasgow pernicious anaemia and Sjögren's syndrome patients were performed by the method described by Anderson et al. (1961a) and in the Washington Sjögren's syndrome patients by Stavitsky's (1954) modification of Boyden's (1951) technique.

Autoimmune complement fixation reaction Tests to detect this factor in the sera of the Washington Sjögren's syndrome patients were made by the method described by Bloch et al. (1960a and b).

A. SYSTEMIC LUPUS ERYTHEMATOSUS

This is the best known syndrome of the "connective tissue disease" group. Clinical diagnosis can be difficult because its manifestations may be protean and it can be misdiagnosed as rheumatoid arthritis or other diseases when the systemic disease is in remission. The L. E. cell test has proved a valuable diagnostic aid since it is frequently positive in systemic lupus erythematosus. There have

been many investigations on other autoimmune phenomena in patients with this disease in attempts to elucidate the underlying immunological abnormality and also to develop improved diagnostic tests.

PATIENTS STUDIED

Serum samples from 80 patients with systemic lupus erythematosus were studied. These patients formed a rather heterogeneous group as the sera had been submitted by numerous physicians, with consequent variation in diagnostic criteria. The incidences of antinuclear antibodies could be compared only with those of precipitating autoantibodies or antibodies to parietal cells of the gastric mucosa, as other laboratory investigations had been undertaken in many hospitals. 46 patients attended hospital in the Glasgow area and 34 in Leeds.

RESULTS

Incidence of Antinuclear Antibodies

These antibodies were found in 66 (82.5%) of the 80 systemic lupus erythematosus patients tested (Table 8/2) whereas the incidence in a series of healthy controls and iron-deficiency anaemia patients matched for age and sex was 5/80 (6.3%). This is, of course, highly significant ($\chi^2 = 91.1$: $P < 0.001$). The antibodies were somewhat commoner in the Leeds patients (30/34) than in the Glasgow patients 36/46 (fig. 8/1); the difference was not significant ($\chi^2 = 0.7$: $P > 0.3$), but it probably reflected the strict adherance to the M.R.C. diagnostic criteria for systemic lupus erythematosus in Dr. Rowell's

series from Leeds. In general, the titres of antinuclear antibodies in systemic lupus erythematosus patients were high as three-quarters of the sera stained nuclei at 1:64 dilution and half were still effective at titre 1:256 (fig. 8/1).

The incidence of various types of antinuclear antibody in patients with systemic lupus erythematosus is shown in Table 8/2. The incidence of the "homogeneous" antinuclear antibody was twice as great as that of the "speckled" antinuclear antibody in the whole series (H:S ratio 2.1) and although there were differences between the H:S ratios in the Glasgow and Leeds groups these are not significant ($\chi^2 = 0.03$: $P > 0.8$). The titres of the different types of antinuclear antibody are shown in fig. 8/2. The "homogeneous" sera (33/43 had titre 1/64 or 1/256) were in general not so strong as the "speckled" sera (15/20 had titre 1/256 or 1/1000). Antinucleolar antibody was seen only twice; in each case, it was present in low titre and was associated with low titre "homogeneous" antibody. The "membranous" antinuclear antibody was found in 3 Glasgow patients, all of whom were in the terminal stages of the disease.

The Relationship of Antinuclear to Precipitating Autoantibodies

The incidence of precipitating autoantibodies detected by Dr J. R. Anderson in this series of systemic lupus erythematosus patients is shown in Table 8/3. Although there were differences between the incidences in Glasgow and Leeds sera, these were not significant ($\chi^2 = 0.03$: ~~$P < 0.8$~~ $P > 0.8$). The only outstanding difference in the

distribution of different precipitin systems was the absence of anti-DNA from the Leeds group. There was no overall relationship between the results of the fluorescent antibody test for antinuclear antibodies and the precipitin test (Table 8/4). The relationship between the different systems of antinuclear antibodies and precipitins is shown in Table 8/5. The only obvious association which was seen was that between the "membranous" antinuclear antibody and anti-DNA precipitin (see Chapter 5). Precipitating antibodies were much commoner in sera containing the "speckled" antibody (75%) than in those with the "homogeneous" antibody (46.5%); this difference was significant at the 1 in 20 level ($\chi^2 = 4.45$: $P < 0.05$).

The Relationship of Antinuclear to Antigastric Antibodies

Antigastric antibodies were detected (jointly with Dr J. R. Anderson) in 6 of the 73 patients tested (8.8%); 5 of the positives were found among 40 Glasgow patients and 1 in 33 Leeds patients.

Although striking, the geographic difference in incidence is not significant ($\chi^2 = 1.06$: $P \fallingdotseq 0.3$). The presence of antigastric antibodies was unrelated to antinuclear antibodies (Table 8/6) or to any other serological abnormality.

Serial Antinuclear Antibody Studies in Systemic Lupus Erythematosus

Duplicate specimens were obtained from almost all the systemic lupus erythematosus patients studied but serial specimens over a period of at least 2 years were obtained from only 6 patients. The findings in these patients are presented in figs. 8/3 - 8/8 where

they are compared with the changes in the E.S.R., the L. E. cell test and the precipitin test for autoantibodies.

There were no dramatic fluctuations in titre of antinuclear antibodies in any of the patients studied despite dramatic clinical remissions in the early period of study of 2 of the patients (figs. 8/3 and 8/4). During remission, the L. E. cell test became negative in two patients (figs. 8/3 and 8/4) but remained positive in two other patients (figs. 8/5 and 8/6). The precipitin test gave a weaker positive during remission in one patient (fig. 8/3) but the reaction became stronger during remission in another patient (fig. 8/4).

Effect of Pregnancy on Antinuclear Antibodies in Systemic Lupus Erythematosus

Two patients, Mrs Ney. and Mrs Dal. were studied during three pregnancies. The clinical histories of these patients are given in appendices 9/1 and 9/2. Serum samples withdrawn during the course of each pregnancy were stored at - 16°C and titrated blindly for antinuclear antibodies in the same batch. The findings are presented in figs. 8/7 and 8/8. Throughout each pregnancy and the subsequent puerperium, the titres of antinuclear antibodies fluctuated only over one dilution step. Since this variation was within the range of experimental error and since there was no consistent trend, it must be concluded that there were no significant alterations in the titre of antinuclear antibody during pregnancy or the puerperium

in these two patients.

Effects of Thymectomy on Antinuclear Antibodies in Systemic Lupus Erythematosus

In view of the enthusiastic recommendation by Sir Macfarlane Burnet to Dr. J. A. Milne (Dermatology Department, Western Infirmary, Glasgow) that thymectomy may have a beneficial effect on systemic lupus erythematosus, a patient with severe renal involvement was subjected to this operation. Her clinical history is summarised in appendix 8/1. Serial samples of serum withdrawn before and after thymectomy were titrated for antinuclear antibody. High titre "speckled" and low titre "homogeneous" antinuclear antibodies were detected in all specimens but no significant change in titre was seen during the period of study (fig. 8/9).

DISCUSSION

Incidence of Antinuclear Antibodies Detected by the Fluorescent Antibody Technique

The published reports (summarised in Table 8/7) have all shown that antinuclear antibodies are very common in systemic lupus erythematosus. The incidence in the present series was lower than that in most previous reports. This could not be attributed to a low sensitivity in my experimental technique as the incidence in controls was comparable to that found in other series. It was possible, however, that the variations in incidence might be attributed to differences in diagnostic criteria used in selection of

patients in different series. Thus, if a positive L. E. cell test was obligatory, antinuclear antibodies will be detected in practically all patients. (The rare exceptions would probably have an antibody which reacted only with mechanically damaged nuclechistone of the type described by Svec and Koplan, 1963). The diagnostic criteria suggested by Bywaters and Ansell (1952) have been widely accepted in this country; they are strict and emphasise the systemic nature of the disease but do not demand a positive L. E. cell test. The Leeds patients were selected on this basis; in this group antinuclear antibodies could not be detected in four patients, all of whom had a negative L. E. cell test. The incidence of antinuclear antibodies was lower in the Glasgow patients (not statistically significant); unfortunately, these sera were submitted from many sources and it is possible that some of the patients were in clinical remission when their serum was taken, or that some did not suffer from systemic lupus erythematosus.

Titres of Antinuclear Antibodies

There have been only three previous quantitative investigations with the fluorescent antibody technique in systemic lupus erythematosus (Alexander et al., 1960; Baugh et al., 1960; Mandema et al. 1961) and in these surveys, most sera had high titre antinuclear antibodies. The present investigation confirmed these findings.

Nuclear Staining Patterns

In this investigation, most of the sera gave "homogeneous"

staining when tested at low titre but on dilution it became obvious that the "speckled" antibody was predominant in 20 of the 66 positive sera. In the only previous survey in which attention was paid to the nuclear staining pattern (Fennel et al., 1962) sera were tested undiluted and, not surprisingly, all sera gave overall staining of the nucleus ("homogeneous" pattern).

Nucleolar staining was seen with only two sera in my series. It was possible that antinucleolar antibody was truly uncommon in systemic lupus erythematosus but this appeared highly improbable in view of the great variety of autoantibodies which have been detected in this disease (Shulman, 1963). It was highly probable that nucleolar staining pattern had been masked by higher titre "homogeneous" or "speckled" antinuclear antibody. There has been a report of antinucleolar antibody in a single case of systemic lupus erythematosus (Pachas et al., 1962).

"Membranous" staining was seen with three sera and this pattern has also been seen by Casals et al. (1963) with systemic lupus erythematous sera.

Other Immunological Tests for Antinuclear Antibodies

There have been many previous investigations using conventional immunological techniques for antinuclear antibodies in systemic lupus erythematosus (Table 8/7). There has been considerable variation between the findings in the different series but, probably, this reflects the differences in the experimental techniques employed.

These results have emphasised the variety of different antinuclear antibodies which may occur in systemic lupus erythematosus and this has been confirmed by the variety of nuclear staining patterns I have seen in sera from patients with this disease.

The results of the present fluorescent antibody survey were compared with those obtained in a precipitin reaction against purified DNA. It was reassuring that the only correlation found was that between "membranous" staining and DNA precipitin as both are considered to be manifestations of anti-DNA antibody (Chapter 5).

Precipitating Antibodies to Tissue Extracts in Systemic Lupus Erythematosus

The precipitating autoantibodies have been shown to be a prominent feature in systemic lupus erythematosus (Anderson et al., 1962). No correlation has been found between the overall incidence of these antibodies and the antinuclear antibodies. It therefore appears that the two systems are unrelated with the exception of the uncommon "membranous" antinuclear sera which contained anti-DNA precipitin. The "membranous" antinuclear antibody was present in the sera of three Glasgow patients in the terminal stages of the disease; it was not found in any of the Leeds sera but none of the Leeds patients were tested when in extremis.

Precipitating autoantibodies were statistically commoner in patients with the "speckled" antinuclear antibody than in those with the "homogeneous" antinuclear antibody. There was no obvious

explanation for this phenomenon. It was possible that the patients with the less common "speckled" antinuclear antibody had a more widespread disturbance of immunological tolerance and this would be supported by the large proportion of these patients who had a precipitin other than anti-SjD, but there was no indication that the prognosis was any poorer in these patients as would have been expected if the autoimmune phenomena were important in the causation of the disease.

Antigastric Antibodies in Systemic Lupus Erythematosus

The incidence of antibodies to the parietal cells of the gastric mucosa was greater in systemic lupus erythematosus patients than in healthy controls matched for age and sex. There was no correlation between antigastric antibodies and other systems of abnormal antibodies but this had not been anticipated as these antibodies have completely different immunological specificities. The incidence of antigastric antibodies encountered in my series of systemic lupus erythematosus patients was much higher than that reported by Doniach et al. (1963).

This difference cannot be attributed to any difference in sensitivity between the fluorescent antibody techniques used in the two laboratories because the incidences observed in controls and in pernicious anaemia patients have been virtually identical in both series (Section G). Furthermore, a difference was noted between the incidences in the Glasgow and Leeds patients in my series. The

incidence in the Leeds patients was comparable with that found by Doniach et al. (1963) whereas the antigastric antibodies were five times commoner in the Glasgow patients. Taken by themselves, the differences between the incidences in the two sub-groups (Leeds and Glasgow) in my series were not statistically significant at the 5% level, but the true significance may be somewhat greater in view of other evidence.

1. Dr. J. R. Anderson (unpublished experiments) has shown that antigastric antibodies are commoner in Sjögren's syndrome patients from Glasgow (13/59) than in patients from Washington (1/39).

2. Antithyroid antibodies were commoner in systemic lupus erythematosus patients from Glasgow (Anderson et al., 1961b) than in patients in the London series (Hijmans et al., 1961).

3. Antithyroid antibodies were commoner in rheumatoid arthritis patients from Glasgow (Buchanan et al., 1961) than in the London series (Hijmans et al., 1961).

4. Antinuclear antibodies were commoner in pernicious anaemia patients from Glasgow (Section G) than in the London series (Doniach et al., 1963).

Direct comparison of results is only valid primary evidence when the tests have been performed under identical conditions in the same laboratory, but comparison between the results of different series can yield valuable supporting evidence. The data presented above suggested that there was a greater tendency for the development of

"accessory" autoantibodies in patients from the West of Scotland. This would be difficult to substantiate unless sera were exchanged between centres. It would also be interesting to study autoimmune disease patients from other areas where the patients are predominantly Scottish in origin, i.e. Nova Scotia in Canada and Dunedin in New Zealand.

Serial Antinuclear Antibody Studies in Systemic Lupus Erythematosus

Only 6 patients were followed for a sufficiently long time to allow observations on the relation of antinuclear and other auto-antibodies to the activity of the disease. No gross alteration in the antinuclear antibody titre was noted in any of these patients during the period of study. It must be explained, however, that steroid and anti-malarial drugs were used only to alleviate the clinical manifestations of the disease and the large dosages which would have been necessary to suppress the disordered immunological phenomena were not employed. This probably explains why the dramatic fall in serum antinuclear activity found by Baugh et al. (1960) and Townes et al. (1963b) was not confirmed in the present investigation.

In general, when the L. E. cell test became negative in my patients the titre of antinuclear antibody fell but the relationship between the two tests is not simple as negative L. E. cell tests were occasionally encountered when the antinuclear antibody titres had risen. Such discrepancies might be explained by errors in the L. E. cell tests, but since this is a complex test requiring in

addition to antinuclear antibody other non-specific factors such as complement (Formijne and van Soeren, 1958) these discrepancies might possibly have resulted from a fall in serum complement which often occurs in exacerbations of the disease (Townes et al., 1963b).

Effect of Pregnancy on Antinuclear Antibody Titre in Systemic Lupus Erythematosus.

The titre of antinuclear antibodies did not alter significantly in three patients during pregnancy or in the puerperium. No other autoantibodies were detected in these women during these pregnancies. There have not been any previous observations on the effect of pregnancy on non-organ-specific autoantibodies in the "connective tissue diseases" but the effect of pregnancy on the clinical manifestations of the disease has been widely studied. Dziubinski et al. (1962) and Ricks (1962) have concluded that pregnancy has little effect on the disease and Donaldson and de Alvarez (1962) state that only 17% of their patients deteriorated during pregnancy or the puerperium but Ellis and Bereston (1952) and Garsenstein et al. (1962) have shown that there may be an appreciable maternal mortality (25% and 20% respectively) usually due to renal failure in the puerperium. Garsenstein et al. (1962) have claimed that exacerbations of the disease are most frequent within two months of delivery. It would be interesting to study the immunological changes in patients who show such clinical deterioration.

Effect of Thymectomy on Antinuclear Antibody Titre in Systemic Lupus Erythematosus

The patient described in appendix 8/1 showed considerable

immediate objective clinical improvement after thymectomy. This could not be attributed solely to medical therapy but the effect of psychological factors has still to be assessed. It is still too soon to assess the effects of the operation on the natural history of the disease. The operation had no effect in this short term study on the titre of antinuclear antibody (or on the precipitating autoantibodies detected by Dr J. R. Anderson).

It seems highly probable that the half life of antinuclear antibody would be substantially less in this patient than in the healthy babies described in Chapter 9, because there would be considerable release of cellular antigens from the tissues damaged by the lesions of active systemic lupus erythematosus. If that were so, the titre of antinuclear antibody should have fallen dramatically if there had been a substantial diminution in the production of these antibodies after thymectomy. As the titre of antinuclear antibody has not changed after the operation it must be concluded that the production of antinuclear and presumably also of the precipitating autoantibodies had not been affected by thymectomy. Although it was not possible to assess the effect of this procedure on any abnormal delayed hypersensitivity reactions (and these are almost certainly present in this disease) it seems highly probable that these would also have remained unaffected by thymectomy.

There has been one previous report of thymectomy in systemic lupus erythematosus (Mackay et al., 1963), and in this case the

post-operative improvement could have been attributed to the increase in steroid dosage. The L. E. cell test was positive before the operation and this abnormality persisted afterwards; then, as in the case reported above, thymectomy did not interfere substantially with the production of antinuclear antibody. Larrson (1963) has described a patient in whom systemic lupus erythematosus developed in association with a thymoma. Weak antinuclear antibodies were demonstrated by the fluorescent antibody technique in serum obtained two weeks after thymectomy but subsequent specimens were negative. It would not be reasonable to conclude that this patient showed a post-operative fall in titre of antinuclear antibody as weak reactions are very difficult to assess (Chapter 8) and are seldom regularly reproducible.

Burnet (1963) has suggested that the thymus may be the site of origin of "forbidden clones" and he has suggested that thymectomy at an early stage might prevent further progression of the disease. It seems unlikely that it will ever be possible to remove the thymus at a sufficiently early stage since Alarcon-Segovia et al. (1963) have reported two patients who developed systemic lupus erythematosus six years and three years after thymectomy for myasthenia gravis.

SUMMARY

Strong antinuclear antibodies were demonstrated in the sera of 66 of 80 patients (82.5%) with systemic lupus erythematosus and in

5 of 80 matched controls (6.3%). The "homogeneous" antinuclear antibody was the dominant antibody in 43 sera, the "membranous" in 3 sera, the "speckled" in 20 sera and the antinucleolar antibody in 2 sera. There was no relationship between the systems of antinuclear antibodies or their titres and the activity of the disease or the presence of other systems of autoantibodies. No significant change in the antinuclear antibody titre was detected in 2 patients during 3 pregnancies nor after thymectomy in 1 patient.

B. DISCOID LUPUS ERYTHEMATOSUS

There is now little doubt that systemic lupus erythematosus and discoid lupus erythematosus are variants of the same fundamental disease. The older clinical and morbid anatomical literature has been reviewed by Harvey et al. (1954) and Gold (1960) and recently patients showing a change from discoid lupus erythematosus to the systemic disease have been described by Storck and Berzups (1962) and vice versa by Ganor and Sagher (1962). In addition, there have been reports of haematological abnormalities (Scott and Rees, 1959; Marten and Blackburn, 1961) and plasma protein changes (Gold, 1960) in discoid lupus erythematosus; these findings were similar to those commonly found in systemic lupus erythematosus, but less intense. It was therefore decided to investigate the sera of patients with discoid lupus erythematosus for antinuclear antibodies since these antibodies have been shown to be very common in the systemic disease.

PATIENTS STUDIED

Sera from 137 patients with discoid lupus erythematosus were tested for antinuclear antibodies; 120 patients were attending Dr N. R. Rowell at the General Infirmary, Leeds and the other 17 were being treated in the Glasgow area. The Leeds patients were consecutive out-patients with typical discoid lupus erythematosus (confirmed by biopsy in most cases) and no evidence of systemic involvement on clinical examination.

RESULTS

Incidence of Antinuclear Antibodies

When the sera of 137 patients with discoid lupus erythematosus were tested for antinuclear antibodies by the fluorescent antibody technique, 48 sera (35.0%) gave nuclear staining at dilution 1:16, whereas the incidence in a series of healthy controls and iron deficiency anaemia patients matched for age and sex was 12/137 (8.8%). The difference was highly significant ($\chi^2 = 26.14$; $P<0.001$). The incidence was the same in the Leeds' patients (42/120, i.e. 35%) and in the remainder (6/17, i.e. 35%). In the Leeds sera there were minor differences between the incidences in males (10/37, i.e. 27%) and females (32/83, i.e. 38.5%) but these were not significant ($\chi^2 = 0.0035$; $P = 0.95$) (Table 8/8). Although 27 of these sera had low titre (1/16) antinuclear antibodies, 20 had a titre of 1:64 or greater and one serum had titre 1:1,000 (fig. 8/10). The distribution of titres in the Leeds sera and the remainder was similar. The "homogeneous" antinuclear antibody was about twice as

common as the "speckled" antinuclear antibody in the whole series (H:S ratio = 2.2) and in both groups, and similar ratios were seen in male and female Leeds patients (Table 8/8). The incidence of titres of the different antinuclear antibodies is shown in fig. 8/11. The single antinucleolar antibody had a titre of 1:64 and was found in the miscellaneous group of patients collected at Glasgow.

Relationship of Antinuclear Antibodies to other Findings

The clinical and laboratory findings in the Leeds discoid lupus erythematosus patients are summarised in Table 8/9, which shows that, apart from Raynaud's phenomenon, anaemia and possibly also leucopenia, there is no marked sex bias in these results. The incidence of antinuclear antibodies in patients with other abnormalities is shown in Table 8/10, which includes results of the statistical analysis of the findings.

(a) Sex of patient Antinuclear antibodies were more common in female patients than in males (32/83 compared with 10/37) but the differences were not significant ($\chi^2 = 1.03$; $P = 0.3$). There was no difference in the distribution of the different types of antinuclear antibody as the H:S ratio was 22:10 in females and 7:3 in males.

(b) Age The incidence of antinuclear antibodies in patients in different age groups is shown in fig. 8/12, which shows that the antibodies were commoner in older patients.

(c) Duration of disease The incidence of antinuclear antibodies

was higher in patients who have had the disease for longer periods (fig. 8/13).

(d) Extent of skin involvement This was assessed from the number of areas (face, scalp, arms, etc.) affected by the disease. Fig. 8/14 shows that antinuclear antibodies were much commoner when the disease involved more than one area ($\chi^2 = 5.94$; $P \doteqdot 0.02$).

(e) Chilblains Although the incidence of antinuclear antibodies was raised in patients with chilblains (Table 8/10), the distribution of different types of antibody was the same as that found in the remainder of the discoid lupus erythematosus patients (Table 8/11).

(f) Raynaud's phenomenon The incidence of antinuclear antibodies was raised in patients showing Raynaud's phenomenon (Table 8/10) and although the "speckled" antinuclear antibody was commoner in these patients, the difference in distribution of antibody types was not significant (Table 8/12).

(g) Joint pains Patients complaining of joint pains showed a raised incidence of antinuclear antibodies (Table 8/10). There was a disproportionately high incidence of "homogeneous" antinuclear antibody in these patients (Table 8/13) but the observed distribution of antibody types did not differ significantly from that found in patients who did not complain of joint pains ($\chi^2 = 1.35$; $P \doteqdot 0.2$).

(h) L.E. cell test This test was consistently negative in 118 patients. It was intermittently positive in the other 2 patients both of whom showed "homogeneous" antinuclear antibody (titre 1/64)

in serum withdrawn at the same time. It is noteworthy that the "homogeneous" antibody was present in 27 sera which were negative in the L. E. cell test.

(i) Rheumatoid factor Antinuclear antibodies were commoner in patients whose Rose-Waaler test was positive at titre 1:16 than in those patients who were negative or had lower titres (Table 8/10). The "speckled" antinuclear antibody was much commoner in patients with a positive Rose-Waaler test but the difference in the distribution of antinuclear antibody types from the Rose-Waaler negative patients was not significant (Table 8/14). There is some correlation between the titres of antibodies detected by the two systems but Table 8/15 confirms that the two tests measure different antibodies as some sera showed a high titre in one test but were negative in the other.

(j) Precipitating autoantibody Four patients, whose clinical histories are given in appendix 8/2 and summarised in Table 8/16, had anti-SjT precipitating antibody in their serum and in addition, each had "speckled" antinuclear antibody and rheumatoid factor. These four patients were distinguished from the remainder of the discoid lupus erythematosus series as each had had, in addition, episodes of annular lesions (fig. 8/15) resembling erythema multiforme on the arms and legs and less frequently on the face, neck, chest and in the mouth. These lesions were present for relatively short-periods (a few days to over a month) and their onset could not be related to

the season of the year or to any other provoking cause. Episodes have recurred at intervals over a course of up to 20 years. Early lesions start as erythematous papules but the well-developed lesion takes the form of a ring, the edge of which may be vesicular. Healing may occur without scarring, but if the reaction is more intense, bullae form, and even necrosis and ulceration may develop. Histology of the lesions (reported at the Pathology Department, the University of Leeds) showed no striking features but was compatible with erythema multiforme. All the patients suffered from erythrocyanosis of the legs and chilblains and the toes and sides of the feet presented erythema corresponding to what has been described as "Hutchinson's chilblain lupus". Arthritis was not a feature although one patient had occasional pain in a shoulder and another in an ankle. Most episodes were afebrile.

The results of investigations are summarised in Table 8/16. The erythrocyte sedimentation rate was raised in 2 of the 4 patients, the serum globulin was raised in 3 and there was slight anaemia, leucopenia and thrombocytopenia in 2 patients. These findings do not necessarily indicate a diagnosis of systemic lupus erythematosus as such abnormalities were found in 55% of the Leeds series of apparent discoid lupus erythematosus. The negative L. E. cell tests in all 4 patients were not surprising as I have never demonstrated the "homogeneous" antinuclear antibody, which is probably identical with the L. E. cell factor (Chapter 2), in the patients with ringed

lesions. Antimalarial drugs do not seem to influence the toxic lesions. One of the patients has had no further lesions since starting on systemic steroids but in another this treatment has had no effect. The antibody titres have not altered markedly in any of the patients over periods of observation of up to three years.

DISCUSSION

(a) Incidence of Antinuclear Antibodies in Discoid Lupus Erythematosus These antibodies were detected in the sera of 48 of the 137 consecutive cases studied above (35%). This incidence is much lower than that encountered in systemic lupus erythematosus (82%), progressive systemic sclerosis (81%) and Sjögren's syndrome (62%) but significantly higher than that seen in rheumatoid arthritis (24%) ($\chi^2 = 3.318$; $P \div 0.05$). My findings in discoid lupus erythematosus are somewhat higher than those reported by Weir et al. (1961) (13%) and lower than those recorded by Peterson and Fusaro (1963) (60%) and Shrank and Doniach (1963) (50%). Such differences may reflect the diagnostic criteria used in the selection of patients as Weir et al. (1961) did not include patients with any clinical or haematological evidence of systemic involvement, but differences in the sensitivity of the experimental techniques used in different centres may be important. Peterson and Gökcen (1962) claimed to have found antinuclear antibodies in 28 of 34 cases of chronic discoid lupus erythematosus (82%) whereas only 4% of their controls were positive; these findings must be suspect as these authors state that

they stained human blood films for 12 to 15 hr at 4°C, a technique which in my hands yielded a very high incidence of positives in control sera. Surprisingly, Thivolet and Kratchko (1963) failed to detect antinuclear antibodies in any of 34 patients with discoid lupus erythematosus, although they demonstrated these antibodies in 90% of their patients with the systemic disease; it would be difficult to explain these findings by any difference in the sensitivity of their experimental technique from that employed by other research workers.

Using a sensitive precipitin technique, Bennett et al. (1961) demonstrated anti-DNA in 10 of 16 patients labelled discoid lupus erythematosus but these patients showed a high incidence of other abnormalities so that the series must have included some patients with systemic involvement. It was not possible to compare these results with the present series.

(b) Titre of Antinuclear Antibodies in Discoid Lupus Erythematosus

In my series, the titres of antinuclear antibodies in discoid lupus erythematosus were, in general, lower than those found in systemic lupus erythematosus and progressive systemic sclerosis. Comparable titres were found in Sjögren's syndrome, but the rheumatoid arthritis sera showed a preponderance of low titres (Section E). It was not possible to compare these results with the findings in previous fluorescent antibody investigations as none of these have been quantitative.

(c) Types of Antinuclear Antibodies in Discoid Lupus Erythematosus

The ratio of the incidence of "homogeneous" to "speckled" antinuclear antibodies was 2.2 in discoid lupus erythematosus. This value was identical with that found in systemic lupus erythematosus and progressive systemic sclerosis and contrasted markedly with the values of 1.4 and 5.0 found in Sjögren's syndrome and rheumatoid arthritis respectively. It is not possible to compare the results with the findings of Weir et al. (1961), Peterson and Fusaro (1963) and of Shrank and Doniach (1963) as these authors did not differentiate the systems of antinuclear antibodies in their papers.

Peterson and Gökcen (1962) claimed to have detected "speckled" staining with 4 of their 28 positive sera, but it is difficult to interpret this paper as they did not indicate which cell nuclei showed "speckled" staining. If they had made this observation on all cell nuclei, it would certainly have been an artefact as it will be shown in Chapter 11 that the "speckled" antigen is absent from the polymorph nuclei and is restricted to the nuclei of lymphocytes and monocytes in normal blood.

The antinucleolar antibody was seen in my series only once and is therefore very rare. Peterson and Gökcen (1962) claim to have seen this antibody in one of their 28 positive sera but no morphological details were given in their paper. Their observations could not be accepted unless it was restricted to lymphocytes and monocytes as it will be shown in Chapter 11 that nucleoli are present

in normal blood only in these cell types.

The "membranous" antinuclear antibody was not detected in my series of discoid lupus erythematosus patients. This was not surprising as this antibody has been found in systemic lupus erythematosus only during severe clinical relapse.

(d) Relationship of Antinuclear Antibodies to Clinical Findings in Discoid Lupus Erythematosus

(i) Sex In view of the higher incidence found in the women in a series of random hospital patients (Chapter 7), it was interesting that antinuclear antibodies were commoner in women in this series. However, the sex difference was not significant at the 1 in 20 level, so my findings agree with the previous report of equal sex incidence (Shrank and Doniach, 1963).

(ii) Age and duration of disease The increased incidence of antinuclear antibodies in older patients probably reflected the increased tendency to produce these antibodies which was suggested by the raised incidence in older subjects both in the control group and the survey of random hospital patients recorded in Chapter 7. The increased incidence of antinuclear antibodies with disease of longer duration was probably dependent upon the older age of these patients. Shrank and Doniach (1963) did not note any relationships to age or duration of disease.

(iii) Extent of skin involvement Antinuclear antibodies were commoner in patients who had more than one region of the body

affected by discoid lupus erythematosus. A previous report (Shrank and Doniach, 1963) showed a similar trend, but the differences were not significant. There was no direct relationship between antinuclear antibodies and the skin lesions, as occasional patients with severe skin lesions did not have antinuclear antibody and vice versa, and this was compatible with other observations (Chapter 9) that antinuclear antibodies were probably not important in the pathogenesis of disease.

(iv) Chilblains and Raynaud's phenomenon Antinuclear antibodies were common in patients with these symptoms. The incidence of these antibodies is being investigated currently in patients with "idiopathic" chilblains and Raynaud's phenomenon to determine whether these abnormalities are important by themselves or merely as indicators of mild systemic involvement.

(v) Joint pains In one case, the joint pains can be explained by rheumatoid arthritis which was present as a complication of discoid lupus erythematosus, but organic joint disease was not diagnosed in the remainder of the patients. Joint involvement is common in systemic lupus erythematosus and it is possible that joint pains may indicate some degree of systemic involvement in patients with discoid lupus erythematosus. This possibility is given more weight by the raised incidence of antinuclear antibody in such patients.

There are marked differences in the H:S ratios in patients with joint pains (4.7) and those without joint pains (1.5). The

differences between these values are not significant ($P > 0.2$) with the number of patients studied but they are noteworthy in view of the similar (and also not significant) variations between the H:S ratios for groups A and E Sjögren's syndrome patients (Table 8/21).

(e) Relationship of Antinuclear Antibodies to other Laboratory Findings in Discoid Lupus Erythematosus

(i) Anaemia It was not surprising that anaemia was common in the women in this series, as iron deficiency anaemia is very common in women in the general population. Anaemia and antinuclear antibodies were unrelated in this series.

(ii) Leukopenia and thrombocytopenia Antinuclear antibodies were found in 11 of the 13 patients with these haematological abnormalities in this series (Table 8/10) and Shrank and Doniach (1963) have demonstrated antinuclear antibodies in each of the 7 patients with haematological abnormalities in their series. It is probable that this association of antinuclear antibodies with leukopenia and thrombocytopenia has resulted from low grade systemic involvement as there is no evidence that the antinuclear antibodies can act on circulating cells *in vivo* (Chapter 9).

(iii) Erythrocyte sedimentation rate Antinuclear antibodies were commoner in patients with a raised E.S.R. than in the remainder of this series. This association has not been previously recorded but presumably it reflects low grade systemic involvement with alteration of plasma proteins.

(iv) Alterations in plasma proteins The incidence of antinuclear antibodies in patients with disturbances of the plasma proteins was much greater than in the remainder of the series. This relationship has not been recorded previously but it was not unexpected as raised γ globulin levels are probably produced, at least in part, by the circulating autoantibodies.

(v) L. E. cell test Both patients with positive L. E. cell tests had "homogeneous" antinuclear antibody. This was anticipated as both reactions have been shown to demonstrate antinucleohistone (Chapter 2).

It was noteworthy that many other patients had this antibody in their serum but had negative L. E. cell tests. Most of these sera had low titre "homogeneous" antinuclear antibody which would not have been detected by the insensitive L. E. cell test. In the others, low serum complement (which is frequent in systemic lupus erythematosus, Williams and Law, 1958; Ellis and Felix-Davies, 1959) might explain the negative L. E. cell tests as Formijne and van Scoyen (1958) have shown that complement is necessary for the L. E. cell phenomenon.

(vi) Rheumatoid factor Antinuclear antibodies were commoner in patients with significant titres of rheumatoid factor than the remainder of the series. There was no correlation between the titres of the two tests, but this was not expected as the antibodies are known to react with separate antigens. This association has

not been recorded previously, but presumably it reflects the increased tendency to produce autoantibodies in patients with low grade systemic involvement.

(f) Anti-SjT Precipitating Autoantibodies and the Syndrome of Erythema Multiforme-like Lesions in Discoid Lupus Erythematosus

The four discoid lupus erythematosus patients with anti-SjT precipitating autoantibody all had "speckled" antinuclear antibody and rheumatoid factor; furthermore, all had had episodes of erythema multiforme-like lesions. It was important to decide whether these cases were patients with discoid lupus erythematosus who had developed erythema multiforme incidentally or whether this was a distinct syndrome. The association of similar clinical and immunological patterns in 4 patients made it unlikely that this was a spurious association, especially as erythema multiforme has not (in my experience of 12 cases) been associated with antinuclear or precipitating autoantibodies, nor have anti-SjT precipitating antibodies been found in uncomplicated discoid lupus erythematosus patients. Moreover, there does not appear to be any specific initiating factor (such as drug-taking or infection) to account for the episodes of erythema multiforme.

The suggestion that the association of the clinical and immunological manifestations in these 4 patients constituted a true syndrome (Rowell et al., 1963) was further supported by recent investigations on sera from another two women with discoid lupus

erythematosus with associated annular erythema multiforme since I have shown that these new patients also had "speckled" antinuclear antibody, rheumatoid factor and anti-SjT precipitin - the same immunological defect as the four cases described above. A clinical report on one of these patients (Mrs. Chi.) has been presented at a recent meeting of the Royal Society of Medicine (Munro, 1963). The other (Mrs. Sam.), a patient from Grimsby, also had anti-SjD precipitin in her serum. In contrast, a woman (Mrs. Cle.) with discoid lupus erythematosus and blotchy toxic erythema (quite unlike the ringed lesions) had anti-SjD and weak anti-Lup precipitins but I did not demonstrate antinuclear antibodies in her serum.

The association of lupus erythematosus and erythema multiforme has been reported previously. Scholtz (1922) described a case of a woman of 31 years, who had discoid lupus erythematosus of the face and chest. She later developed a butterfly rash on the face and maculo-papular lesions, like the papular type of erythema multiforme, on the arms, legs, gluteal region and hard palate. The lesions on the face and sternum became haemorrhagic and oozed blood. The lesions began to clear after a week, during treatment with quinine hydrochloride and calcium lactate by mouth, and eventually disappeared completely. She later died from anaemia, and pyonephrosis was found at necropsy.

Ormsby and Montgomery (1943) cited two fatal cases of systemic lupus erythematosus with erythema multiforme-like lesions. The

cases described in appendix 8/2 do not fit into this more fulminating type of lupus erythematosus but in view of the immunological associations and clinical overlap between discoid and systemic lupus erythematosus, it is interesting that I have found almost identical immunological abnormalities in a patient (Mrs Nic.) where erythema multiforme-like lesions complicated systemic lupus erythematosus. This woman, a patient from Manchester, had antinuclear antibodies ("homogeneous" titre 1/1,000 and "speckled" 1/64) rheumatoid factor and weak unidentified precipitins. The presence of high titre "homogeneous" antinuclear antibody was hardly surprising as she had a strongly positive L. E. cell test.

In many of the "connective tissue diseases" several abnormal antibodies to tissue constituents may be present in the serum, but most of them are not specific to any one disease or tissue. In the cases described above, a distinctive clinical picture was associated with a characteristic pattern of immunological abnormality. This was the first report of a constant abnormal immunological response in one of the "connective tissue diseases". It was considered that this association was sufficiently characteristic to justify regarding it as a syndrome. There was, however, no information which would suggest that the prognosis of patients with this syndrome would be materially different from that of uncomplicated discoid lupus erythematosus.

SUMMARY

Antinuclear antibodies were detected in the sera of 48 of 137 patients (35%) with discoid lupus erythematosus and in 12 of 137 matched controls (8.8%). The "homogeneous" antinuclear antibody was the dominant antibody in 33 sera, the "speckled" in 15 sera and the antinucleolar antibody in 1 serum.

In general, the antibody titres were lower than those found in systemic lupus erythematosus but the H:S ratios were similar in both varieties of the disease.

There was no relationship between antinuclear antibodies and the sex of the patient or the presence of anaemia. The antinuclear antibodies were commoner in older patients and in those who had suffered from the disease for longer periods or had more extensive skin involvement. Antinuclear antibodies were commoner in patients who showed some other evidence of low grade systemic involvement, e.g. chilblains, Raynaud's phenomenon, joint pains, leucopenia, thrombocytopenia and raised E.S.R. or plasma γ globulin. Although antinuclear antibodies were more frequent in patients with a positive Rose-Waaler test, the titres of the two systems were quite unrelated.

Four of the patients had ringed erythema multiforme-like lesions and the serum contained "speckled" antinuclear antibody, anti-SjT precipitin and rheumatoid factor in each case. It was considered that this represents a true syndrome rather than a spurious association of two unrelated diseases. There was no indication that the prognosis in these patients is materially different from that of uncomplicated discoid lupus erythematosus.

C. PROGRESSIVE SYSTEMIC SCLEROSIS

There is strong evidence that progressive systemic sclerosis should be classified in the "connective tissue disease" group because of the clinical and morbid anatomical relationships with the other diseases and the frequent occurrence of autoimmune phenomena, such as rheumatoid factor, L. E. cells or other antinuclear antibodies (Rodnan, 1963).

PATIENTS STUDIED

The diagnosis of progressive systemic sclerosis has been accepted only when the patient showed acrosclerosis with systemic changes; patients with only localised cutaneous scleroderma (morphoea) or generalised cutaneous scleroderma without systemic changes have not been included in this series.

Sera from 32 patients were tested for antinuclear antibodies; 22 were attending Dr N. R. Rowell at the General Infirmary, Leeds and the other 10 were being treated in the Glasgow area.

RESULTS

Incidence of Antinuclear Antibodies

Antinuclear antibodies were detected in the sera of 25 of the 32 patients with progressive systemic sclerosis (78%), and in 2 of 32 matched controls (6.3%). The difference was highly significant ($\chi^2 = 31.0$: $P < 0.001$). The incidences in the Glasgow and Leeds patients were virtually identical ($\chi^2 \doteq 0.08$: $P > 0.7$). In general,

the titres of antinuclear antibodies in progressive systemic sclerosis were high as 23 of the 32 sera stained nuclei at 1:64 dilution and 20 were effective at titre 1:256 (fig. 8/16).

The incidence of the different systems of antinuclear antibodies is shown in Table 8/18. The incidence of the "homogeneous" antinuclear antibody was twice as great as that of the "speckled" antibody (H:S ratio 2.1) in the whole series. The minor differences between the Leeds and Glasgow patients were not significant. The titres of the different systems of antinuclear antibodies are shown in fig. 8/17. As in systemic lupus erythematosus, the "homogeneous" sera (14/17 had titre 1/64 or 1/256) were in general not quite as strong as the "speckled" sera (7/8 had titre 1/256 or 1/1000).

The antinucleolar antibody was detected in 6 sera (19%), usually in high titre. In 5 cases, it was associated with the "homogeneous" antibody and in the sixth with the "speckled" antibody. The "membranous" antibody was not detected in any of the sera.

The presence or absence of antinuclear antibody was unrelated to the age or sex of the patients or to the duration or clinical severity of the disease. Examination of sera taken at intervals up to 2 years has shown remarkable constancy of the findings in individual patients. No regular association was found between the presence of antinuclear antibody and raised erythrocyte sedimentation rate, serum globulin greater than 3.0 g./100 ml. or results of Wassermann reaction, Rose-Waaler test, liver function tests or tests

for cryoglobulins or cold agglutinins.

L. E. cells had been demonstrated in 3 of these patients (Rowell, 1962); one showed no evidence of systemic lupus erythematosus, either clinically or at autopsy, in the second there was a previous history of subacute rheumatoid arthritis, whilst it is probable that systemic lupus erythematosus and progressive systemic sclerosis coexisted in the third case. The sera of these three patients produced "homogeneous" nuclear staining, but this is hardly surprising since it has been shown in Chapter 2 that both tests detect the presence of antinucleohistone. Since the fluorescent antibody test is a much more sensitive method for the detection of this antibody, it is not surprising that "homogeneous" nuclear staining was detected in the sera of a further 14 patients in whom the L. E. cell test was negative.

The Relationship of Antinuclear to Precipitating Autoantibodies

The incidence of precipitating autoantibodies detected by Dr J. R. Anderson in this series of progressive systemic sclerosis patients is shown in Table 8/19. Six of the 32 patients had these antibodies: 4 had anti-SjD, one of these had also anti-Lup and another had a second unidentified antibody: 1 patient had anti-Lup alone and one patient had a single unidentified antibody. All of these patients' sera contained antinuclear antibodies but no relationship was apparent between the types of precipitating autoantibody and the pattern of nuclear fluorescence (Table 8/20). Precipitins were

much commoner in patients with "speckled" antinuclear antibody (4/8) than in those with the "homogeneous" antibody (2/17).

It is noteworthy that 5 of the 10 Glasgow patients had precipitating autoantibodies whereas only 1 of the 22 Leeds patients showed this type of antibody (Table 8/19). The two groups of patients were apparently selected with similar diagnostic criteria and showed a similar range of clinical severity of the disease. There is good evidence that the precipitating autoantibodies are not affected by the conditions of storage and transport to which they were subjected. Nor is the difference in incidence of precipitating antibodies explained entirely by the necessity to test the Leeds sera at an initial dilution of 1 in 4, for 4 of the 5 positive Glasgow sera had precipitin titres of 1 in 16 to 1 in 128.

DISCUSSION

Incidence of Antinuclear Antibodies in Progressive Systemic Sclerosis

There have been several sporadic reports of positive L. E. cell tests in patients with progressive systemic sclerosis (see Rowell, 1962) and Corcos et al. (1961) have demonstrated, by a complement fixation technique, antibodies to calf thymus nuclei in 9 of 25 patients.

The fluorescent antibody technique has been used previously to test several small groups of progressive systemic sclerosis patients, but with one exception (Fennell et al., 1962) differences in nuclear staining patterns were not recorded, so that only the overall incidence can be compared with my series. My findings (25/32)

confirm the high incidence reported in certain series (4/6 Bardawil et al., 1958; 2/3 Alexander et al., 1960; 8/10 Hall et al., 1960; 22/27 Fennell et al., 1962). Other authors have found a much lower incidence (3/11, Mandema et al., 1961; 1/5 Thompson, 1962) or failed to detect antinuclear antibodies in patients with "scleroderma" (Kratchko, 1961; Weir et al., 1961); it is possible that these patients suffered from cutaneous scleroderma rather than systemic disease, since I have found low titre antinuclear antibodies in 1 of 3 cases of generalised cutaneous scleroderma and 2 of 9 cases of Morphoea.

Consideration of the patterns of nuclear staining encountered in my series has shown that the H:S ratios were similar in both progressive systemic sclerosis and systemic lupus erythematosus, whereas nucleolar staining was common in progressive systemic sclerosis (6/32) but uncommon in systemic lupus erythematosus (2/80). There is no published report to compare with my observations on the staining patterns of the nucleoplasm but my observation of a high incidence of nucleolar staining confirmed the previous report of nucleolar staining with 3/27 progressive systemic sclerosis sera (Fennell et al., 1962) and with isolated cases of "scleroderma" (Pachas et al., 1962).

Titration of the antinuclear antibodies has shown that high titre antibodies of the three common systems ("homogeneous", "speckled" and "nucleolar") were frequent in my series of progressive

systemic sclerosis patients. High titre antinuclear antibodies have also been found frequently in my series of systemic lupus erythematosus and to a lesser extent in Sjögren's syndrome whereas the sera of patients with discoid lupus erythematosus and rheumatoid arthritis usually contained much weaker antinuclear antibodies or were completely negative. This quantitative assessment confirms the similarity between the immunological disturbance in progressive systemic sclerosis and systemic lupus erythematosus suggested by the H:S ratios; this was unexpected in view of the great dissimilarity between the clinical and pathological features of the two diseases.

Precipitating Antibodies to Tissue Extracts in Progressive Systemic Sclerosis

The incidence and titres of the precipitating antibodies in my series provided a further link between progressive systemic sclerosis and systemic lupus erythematosus, for although anti-SjD has been found in examples of all the various connective tissue diseases, anti-Lup has been detected only in systemic lupus erythematosus and progressive systemic sclerosis. The difference in incidence of precipitins in the Leeds and Glasgow patients could not be explained. It was noteworthy that precipitins were much commoner in patients with "speckled" antinuclear antibody than in those with the "homogeneous" antinuclear antibody as a similar association has been seen in systemic lupus erythematosus.

SUMMARY

Antinuclear antibodies were detected in the sera of 25 of 32 patients (78%) with progressive systemic sclerosis and in 2 of 32 matched controls (6.3%). The "homogeneous" antinuclear antibody was the dominant antibody in 17 sera, the "speckled" in 8 sera and the antinucleolar antibody in 6 sera. In general, the antibody titres were high. The antinuclear antibody production in progressive systemic sclerosis was remarkably like that in systemic lupus erythematosus both in the frequency of high titres and the H:S ratios, but it differed in the high frequency with which antinucleolar antibody was found in progressive systemic sclerosis.

Precipitating antibodies were much more frequent in patients with "speckled" antinuclear antibody but no correlation has been found with particular systems of precipitins.

D. SJOGREN'S SYNDROME

There is little doubt that this syndrome should be classified with the "connective tissue diseases" but it is much more benign than the other diseases of this group and in addition organ-specific lesions of lachrymal and salivary glands (which are reminiscent of those seen in Hashimoto's thyroiditis or pernicious anaemia gastritis) are invariably present. The patients frequently show evidence of non-organ-specific autoimmunity such as antinuclear antibodies but organ-specific autoimmunity has never been detected (Bloch and Bunim, 1963).

Serum samples from 77 patients with Sjögren's syndrome have been studied; 42 were attending the National Institutes of Health, Washington and the remaining 35 were being treated in the Glasgow area.

DIAGNOSTIC CRITERIA

In the National Institutes of Health (Washington) series, all the patients showed at least two features of the classical triad of kerato-conjunctivitis sicca, xerostomia and rheumatoid arthritis which constitutes Sjögren's syndrome. These patients were separated into the five clinical groups described by Bunim (1961) namely:

Group A. Sjögren's syndrome associated with classical or definite rheumatoid arthritis (18 cases).

Group B. Sjögren's syndrome associated with possible rheumatoid arthritis (2 cases).

Group C. Sjögren's syndrome associated with scleroderma (3 cases).

Group D. Sjögren's syndrome associated with myopathy (3 cases).

Group E. Keratoconjunctivitis sicca and xerostomia not associated with any other disease (16 cases).

In the Glasgow series the diagnosis was made from the case-history and clinical observation, supported by objective evidence of keratoconjunctivitis sicca, i.e. positive Schirmer and rose bengal tests. Many of the patients had rheumatoid arthritis or other manifestations of Sjögren's syndrome, but insufficient clinical information was available to separate the patients into Bunim's clinical groups.

RESULTS

Incidence of Antinuclear Antibodies

Antinuclear antibodies were detected by the fluorescent antibody technique in the sera of 48 of my total series of 77 Sjögren's syndrome patients (62%) and in 5 of a control series of 77 normal individuals and iron deficiency anaemia patients matched for age and sex. This difference was highly significant ($\chi^2 = 24.16$: $P<0.001$). The antibodies were present in 20 of the 35 Glasgow patients (57%) and 28 of the 42 Washington patients (66%), but the difference between these incidences was not significant ($0.5> P> 0.3$).

In the whole series, 27 sera had "homogeneous" antinuclear antibody and 21 had "speckled" antinuclear antibody. The corresponding figures for the Glasgow and Washington patients are shown in Table 8/21, but the observed differences were not significant ($P = 0.7$). Antinucleolar antibody was seen in 8 patients in the total series (11%). This antibody was found much more commonly in the Washington sera (6 cases) than in the Glasgow sera (2 cases) and this difference, although not significant ($P = 0.1$), was striking.

The maximum titres of antinuclear antibodies encountered in all the sera and those of the different systems of antinuclear antibodies are shown in figs. 8/18, and 8/19, where the findings in the Washington and Glasgow patients are compared; no difference was obvious between the American and British patients.

Incidence of Antinuclear Antibodies in Clinical Groups of Sjögren's Syndrome

Analysis of this type was possible only with the Washington series (Table 8/21) as the clinical data was inadequate in the Glasgow series. Even so, comparison was possible only between Groups A and E because of the small numbers of patients in Groups B, C and D. The overall incidence of antinuclear antibodies was higher in Group E (75%) than in Group A (56%), but this must be attributed to the much higher incidence of "speckled" antinuclear antibody in Group E, as the incidence of "homogeneous" antinuclear antibody was similar in the two groups. Antinucleolar antibody was very frequent in patients in Group E (33%) but was not encountered in Group A, although it was seen in one patient in Group B (possible rheumatoid arthritis).

The maximum titres of antinuclear antibodies encountered in patients in Groups A and E are compared in fig. 8/20 and the titres of the different systems of antinuclear antibodies are shown in fig. 8/21. These histograms showed that higher titres were encountered in Group E in addition to the higher incidence and greater variety of antinuclear antibodies described above.

Relationship between Antinuclear Antibodies and Clinical Features in the Washington Sjogren's Syndrome Patients

No correlation could be seen between the presence and titres of antinuclear antibodies and the age of the patient (fig. 8/22), the

duration of the disease (fig. 8/23) or the presence of keratoconjunctivitis sicca and xerostomia (Table 8/22). There were too few males in the series to allow deductions on the relative incidence in males and females (Table 8/23).

There was, however, a direct relationship with the presence of parotid gland enlargement, as antinuclear antibodies were significantly commoner in patients with parotid gland enlargement ($P < 0.05$) (Table 8/24). There was also a direct relationship between the serum γ globulin level and the presence and titre of antinuclear antibodies in the Washington series (fig. 8/24) and in Groups A and E (Table 8/25).

Relationship between Antinuclear Antibodies and other Autoantibodies in the Washington Sjögren's Syndrome Patients

All patients with positive L. E. cell tests showed high titre "homogeneous" antinuclear antibody but the converse did not hold. There was no direct relationship between the results of the fluorescent antibody test for antinuclear antibodies and other immunological tests such as bentonite flocculation test (Table 8/26) or sensitised sheep cell agglutination test (Table 8/27) for rheumatoid factor, antithyroglobulin (Table 8/28) and the autoimmune complement fixation reaction (Table 8/29) as many sera were positive with one test but negative with another.

Relationship between Antinuclear Antibodies and Precipitating Autoantibodies in the Whole Series of Sjögren's Syndrome Patients

The incidence of precipitating autoantibodies to saline extracts

of human tissue detected by Dr J. R. Anderson is shown in Table 8/30. The overall incidences of antinuclear antibodies detected by the fluorescent technique and the anti-tissue antibodies detected by the Ouchterlony precipitin system are compared in Table 8/31. These figures suggested that there might be a relationship between the results of the two investigations but that the fluorescent antibody test for antinuclear antibodies was more sensitive.

Table 8/32 shows the incidence of different systems of precipitating autoantibodies in sera containing various types of antinuclear antibodies. Precipitins were least common in sera containing "homogeneous" antinuclear antibody. They were found in 17 of 21 sera containing "speckled" antinuclear antibody and are most common (7/8) in sera containing antinucleolar antibody.

It has not been possible to detect any direct relationship between the systems of antinuclear and precipitating antibodies, since the ratio of the number of sera containing anti-SjD to those containing anti-SjT was similar for each system of antinuclear antibody. The most suggestive finding was the presence of anti-SjD in 7 of the 8 sera containing antinucleolar antibody but it was probable that this merely reflected the high incidence of precipitins in Group E as there was no association between the titres of the two systems (fig. 8/25).

DISCUSSION

Relationship of Sjögren's Syndrome to the "Connective Tissue Diseases"

The diagnosis of Sjögren's syndrome is usually based on the

triad of keratoconjunctivitis sicca, xerostomia and rheumatoid arthritis, but it may also be made when any two of these three features are present (Sjögren, 1943). There is now strong evidence that this syndrome should be classified within the "connective tissue disease" group since rheumatoid arthritis may be replaced by systemic lupus erythematosus (Bain, 1960), progressive systemic sclerosis (Harrington and Dewar, 1951; Oblatt et al., 1958; Stava, 1958; Bloch et al., 1960a; Shearn, 1960; Stoltze et al., 1960) or polyarteritis nodosa (Ramage and Kinnear, 1956; Shearn, 1961). The evidence from these clinical associations is further reinforced by the frequency with which autoimmunity can be detected in patients with Sjögren's syndrome, as this is a prominent feature of "the connective tissue diseases". The following autoantibodies have been found in Sjögren's syndrome:- rheumatoid factor (Bloch et al., 1960b), anti-thyroid antibodies (Bunim, 1961; Anderson et al., 1961a; Stoltze et al., 1960), precipitating antibodies to saline extracts of human tissue (Jones, 1958; Anderson et al., 1961b; Crews and Whitfield, 1963), complement fixing autoantibodies (Bloch et al., 1960b; Deicher et al., 1960; Crews and Whitfield, 1963), L. E. cells (Heaton, 1959; Bloch and Bunim, 1963) and antinuclear antibodies (Bloch et al., 1960b; Beck, 1961b; Heaton, 1962; Thompson, 1962; Vanselow et al., 1963).

Antinuclear Antibodies in Sjögren's Syndrome

These antibodies were detected in 48 of the 77 cases studied

above (62%). This incidence was somewhat lower than that encountered in the "connective tissue diseases" with profound immunological disturbance, e.g. systemic lupus erythematosus (82%) and progressive systemic sclerosis (81%) but it was higher than that found in "connective tissue diseases" with relatively mild immunological disturbance, e.g. discoid lupus erythematosus (35%) and uncomplicated rheumatoid arthritis (24%). The antinuclear antibody titres in Sjögren's syndrome showed a range which was intermediate between those in the two main groups of "connective tissue diseases". The H:S ratio was very much lower in Sjögren's syndrome (1.3) than in any of the other "connective tissue diseases". The differences between the incidences of antinuclear antibodies and their titres in the Glasgow and Washington sera (57% and 66.6%) were not marked but the H:S ratio was very much lower in the former group (1.0) than in the latter group (1.5); these results probably reflected differences in the proportions of patients with various clinical groups of Sjogren's syndrome in the two series.

There have been few previous observations on the occurrence of antinuclear antibodies in Sjögren's syndrome and none of them have distinguished the different patterns of nuclear fluorescence. Thomson (1962) and Vanselow et al. (1963) tested small groups of patients and found antinuclear antibodies in two of six and two of five respectively and Heaton (1962) found antinuclear antibodies in 49% of his cases. Bloch and Bunim (1963) investigated 44 patients (some of whom are included in the present Washington series) and they

detected antinuclear antibodies in 31; the incidence in patients with rheumatoid arthritis was 14/25 and 12/13 in patients without evidence of rheumatoid arthritis. Thus the increased incidence of antinuclear antibodies in Group E had been appreciated but the increased variety of antibodies in this group had not been known previously.

Relationship of Antinuclear Antibodies to Clinical Findings

Comparison between the clinical groups was not possible in the Glasgow patients because of the lack of clinical information. In the Washington series, only Groups A and E could be compared because of the small numbers of patients in Groups B, C and D.

Pronounced differences in the antinuclear antibody response were seen between the patients in the two groups:

(a) antinuclear antibodies were commoner in patients in Group E (75%) than in Group A (56%);

(b) higher titres were commoner in Group E than in Group A (Fig. 8/20);

(c) the H:S ratio was lower in Group E (1.4) than in Group A (4.0);

(d) antinucleolar antibody was common in Group E (33%) but absent from Group A.

In the interpretation of these differences, it is important to remember that the diagnosis of Sjögren's syndrome demanded the presence of a severe salivary or lachrymal abnormality when there was no evidence of rheumatoid arthritis or other "connective tissue

disease" so that we might have selected patients with severer disease in Group E. It was not possible therefore to draw definite conclusions from the quantitative differences in incidence or titre between Groups A and E, but the differences in H:S ratios and incidence of antinucleolar antibodies were objective evidence of a qualitative difference between the disturbed immunological responses in Groups A and E.

The antinuclear antibody findings in Group A (Sjögren's syndrome with rheumatoid arthritis) were remarkably similar to those in uncomplicated rheumatoid arthritis as the H:S ratio was high (4.0 and 5.0 respectively), and antinucleolar antibodies were absent from both groups. Antinuclear antibodies were, however, twice as common in rheumatoid arthritis patients with keratoconjunctivitis sicca (56%) as in those without lachrymal or salivary disease (24%).

Antinuclear antibodies were significantly commoner in patients with parotid gland enlargement which presumably indicated severe local lesions and in hypergammaglobulinaemia which probably followed the autoantibody production.

Relationship between Antinuclear Antibodies and Precipitins

The precipitins were much commoner in sera containing antinuclear antibodies than in those without these antibodies. No direct relationship was found between the individual systems detected by these two techniques although precipitins were found frequently in sera with the "speckled" antinuclear antibody (17/21), and the

antinucleolar antibody (7/8) whereas they were less common in the "homogeneous" antinuclear sera (10/27). This confirms the findings in systemic lupus erythematosus and progressive systemic sclerosis.

Precipitins were a conspicuous feature in patients in Group E (13/16) but rare in Group A (1/18) and a greater variety of systems was seen in Group E. This confirmed the impression gained from antinuclear antibody studies that there was a more widespread breakdown of immunological tolerance in the patients in Group E.

Relationship between Antinuclear and other Autoantibodies

All patients with a positive L. E. cell test showed "homogeneous" antinuclear antibody but the converse did not hold - this phenomenon has been discussed in previous sections. It was not surprising that no direct relationship has been found with tests for rheumatoid factor, antithyroglobulin or the autoimmune complement fixation reaction since these antibodies react with different antigens. High titres of antinuclear antibodies were quite commonly associated with high titres of other autoantibodies. It seemed highly probable that this occurred in patients with more severe autoimmunity.

SUMMARY

Antinuclear antibodies were detected in the sera of 48 of 77 patients with Sjögren's syndrome (62%) and in 5 of 77 matched controls (6.5%). The "homogeneous" antinuclear antibody was the dominant antibody in 27 sera, the "speckled" in 21 sera and the antinucleolar antibody in 8 sera. In general, the titres were lower than those

encountered in systemic lupus erythematosus but higher than in rheumatoid arthritis. The H:S ratio was much lower and the anti-nucleolar antibody commoner than in systemic lupus erythematosus.

Antinuclear antibodies were commoner and occurred in greater variety in Bunim's Group E than in Group A, taken in conjunction with the increased incidence of rheumatoid factor, antithyroglobulin, autoimmune complement fixation reaction and precipitins in Group E patients, the findings support the separation of the clinical groups of Sjögren's syndrome.

E. RHEUMATOID ARTHRITIS

This is the commonest syndrome in the "connective tissue disease" group and antinuclear antibodies have been demonstrated previously in a minority of the patients by other workers using the fluorescent antibody technique.

In this investigation, the sera of 103 patients with rheumatoid arthritis have been tested for antinuclear antibodies. The majority of these sera were supplied by Dr J. R. Anderson and had been stored for about 4 years at - 10°C. Clinical details were unfortunately not available so that the incidence of antinuclear antibodies could be compared only with that of precipitating autoantibodies.

RESULTS

Incidence of Antinuclear Antibodies

These antibodies were found in 24 of 103 patients (24%). This

incidence could not be compared with a matched control population because the age and sex of the patients were unknown. In view of my experience with random hospital patients and in the control series for other diseases, it seemed highly probable that less than 10% of a control series would have been positive and thus that the observed incidence in rheumatoid arthritis would have proved higher than in normal individuals but this cannot be substantiated. The titres of the antibodies (fig. 8/26) detected in rheumatoid arthritis sera were in general much lower than in the generalised "connective tissue diseases" such as systemic lupus erythematosus but they were higher than the titres seen in a random hospital population (Chapter 7).

The incidence of various types of antinuclear antibodies in rheumatoid arthritis is shown in Table 8/38. The H:S ratio was 5.0 and no antinucleolar antibodies were found.

The Relationship of Antinuclear to Precipitating Antibodies

Precipitating antibodies were detected in 4 patients' sera by Dr J. R. Anderson. In each case the antibody was classified "anti-R.A." (Anderson, 1963). These were apparently isoantibodies and all four patients had been transfused previously. Three of the patients had "homogeneous" antinuclear antibody (titres 1/1000, 1/16 and 1/16), but the fluorescent antibody test was negative in the fourth serum. As the "homogeneous" antibody reacted with a wide range of human and animal cell nuclei, this antibody was considered quite distinct from anti-R.A.

DISCUSSION

There have been many previous investigations on the incidence of antinuclear antibodies in rheumatoid arthritis by the fluorescent antibody technique. The results of these investigations have been summarised in Table 8/33. The relatively low incidence (24%) detected in the present investigation confirmed most of the previous reports. Alexander et al. (1960), Hall et al. (1960) and Hijmans et al. (1961) have suggested that the incidence is considerably higher and the evidence in these papers did not indicate that they were using particularly sensitive experimental techniques; it is possible that these authors had selected a higher proportion of complicated or active rheumatoid arthritis patients.

There have been two previous quantitative surveys by titration of antinuclear antibodies in rheumatoid arthritis. Alexander et al. (1960) showed that the titres in their series were fairly high although not as high as in systemic lupus erythematosus, but it must be remembered that these authors detected a high overall incidence in rheumatoid arthritis and therefore were probably dealing with a more active group. Mandema et al. (1961) claimed that the titres in rheumatoid arthritis were very much lower than in systemic lupus erythematosus. The results of the present survey were similar to those obtained by Mandema et al. (1961).

There has been only one previous report of patterns of nuclear staining produced by rheumatoid arthritis sera. In this, Fennell

et al. (1962) noted that all sera produced "homogeneous" staining. The present survey confirms the preponderance of "homogeneous" antinuclear antibodies in rheumatoid arthritis.

The precipitins which have been detected in a small proportion of rheumatoid arthritis patients were probably isoantibodies and were certainly unrelated to the antinuclear antibodies. It was, however, interesting that antinuclear antibodies were found in 3 of the 4 sera and this has suggested that these isoantibodies were particularly liable to develop in patients with an upset of immunological tolerance.

SUMMARY

Antinuclear antibodies were detected in the sera of 24 of 103 patients with rheumatoid arthritis (24%). The "homogeneous" antinuclear antibody was the predominant antibody in 20 sera and the "speckled" in 4 sera but the antinucleolar antibody was not detected in any sera. In general, the titres were very low. The H:S ratio was much higher than in the other "connective tissue diseases".

F. POLYARTERITIS NODOSA AND DERMATOMYOSITIS

These uncommon diseases are usually classified in the "connective tissue disease" group, because of overlap in their clinical features and morbid anatomical appearances with the other members of the group. Since there have been sporadic reports of autoimmune phenomena in these diseases, I have tested sera from 9 patients with polyarteritis nodosa and 3 with dermatomyositis for antinuclear antibodies. These

patients were under the care of various physicians in the West of Scotland.

All sera in this group were negative in the fluorescent antibody test for antinuclear antibodies. It was not possible to draw any definite conclusion from these results, because of the small number of patients' sera available for study, but it was fairly certain that antinuclear antibodies were not a prominent feature of these diseases.

The findings in previous investigations have been summarised in Table 8/34. All of these series have been small because of the rarity of the diseases. My results agreed with those published by Kratchko (1961), Fennell et al. (1962) and Thompson (1962). Antinuclear antibodies have been reported sporadically by other investigators. It is quite possible that occasional patients with these diseases develop antinuclear antibodies, but it is also possible that, because of the difficulties in diagnosis, these authors have included, unwittingly, patients with other "connective tissue diseases".

SUMMARY

Antinuclear antibodies were not detected in any of 9 patients with polyarteritis nodosa or 3 patients with dermatomyositis.

G. PERNICIOUS ANAEMIA

It has been established recently that autoimmunity is a prominent feature in pernicious anaemia. Most patients show organ-specific auto-immunity against the stomach, e.g. antibodies to intrinsic factor

in gastric juice (Schwartz, 1960; Jeffries et al., 1962; Taylor et al., 1962) and to the parietal cells of the gastric mucosa (Irvine et al., 1962; Markson and Moore, 1962a; Taylor et al., 1962). The response is not, however, directed solely against the stomach as there is strong evidence of a raised incidence of organ-specific autoimmunity against the thyroid in pernicious anaemia (Irvine et al., 1962; Markson and Moore, 1962b; Doniach et al., 1963). In view of this overlap between gastric mucosa and thyroid auto-immunity, I decided to investigate the incidence of antinuclear antibodies in pernicious anaemia.

PATIENTS STUDIED

The sera of 50 patients with Addisonian pernicious anaemia were supplied by Dr J. F. Adams (Western Infirmary, Glasgow). These patients had been selected on the following diagnostic criteria: macrocytic anaemia, megaloblastic erythropoiesis, low serum vitamin B12 level (< 80 µµg/ml.) and a histamine-fast achlorhydria before treatment. Parenteral cyanocobalamin or hydroxocobalamin produced a satisfactory haematological response in these patients and all had poor intestinal absorption of radioactive cyanocobalamin which was restored to normal by the addition of intrinsic factor.

RESULTS

Incidence of Antinuclear Antibodies

These antibodies were found in 9 of the 50 pernicious anaemia patients' sera (18%) and in 3 of a control series of healthy

individuals and iron deficiency anaemia patients matched for age and sex (6%) (Table 8/35). The difference between the incidences in the two series was marked but it could not be regarded as significant ($\chi^2 = 2.4$: $P \fallingdotseq 0.1$). The titres of the antinuclear antibodies in the pernicious anaemia patients were somewhat higher than those seen in the control series (fig. 8/27). All positive sera in both groups had "homogeneous" antinuclear antibody but one of the pernicious anaemia patients also had antinucleolar antibody. The "speckled" antinuclear antibody was not identified in any of the sera (Table 8/35). Antinuclear antibodies were equally common in males (3/18) as in females (6/32) and showed no predilection for any age group (fig. 8/28).

Relationship of Antinuclear to Antigastric Antibodies

Antigastric antibodies were detected (jointly with Dr J. R. Anderson) in 43 of the patients and in 4 of the controls; antinuclear antibodies were not significantly commoner in the patients with antigastric antibodies than in the remainder ($\chi^2 = 0.065$: $P \fallingdotseq 0.8$) (Table 8/36).

Relationship of Antinuclear to Antithyroid Antibodies

Antithyroglobulin was detected by the tanned cell agglutination reaction by Dr J. R. Anderson in 12 patients. The incidence of antinuclear antibodies in these patients was not significantly greater than in the remainder ($\chi^2 = 0.18$: $0.7 > P > 0.6$) (Table 8/36).

Relationship of Antinuclear Antibodies to Rheumatoid Factor

Rheumatoid factor was demonstrated in 10 patients' sera by the Hyland latex "R.A." test. There was no correlation between this factor and antinuclear antibodies ($\chi^2 = 0.076$; $P \fallingdotseq 0.8$) (Table 8/36).

Relationship of Antinuclear and Precipitating Tissue Antibodies

Dr J. R. Anderson has detected precipitating tissue antibodies in the sera of 7 patients. Three of these sera contained an antibody which was too weak for classification. The other 4 sera all contained an antibody which has been designated anti-K, but it has not yet been decided whether this is an auto- or iso-antibody; one of the sera in this group also contained anti-SjD.

There was no overall correlation between antinuclear and precipitating antibodies ($\chi^2 = 0.0025$; $P \fallingdotseq 0.95$) (Table 8/36). The three sera with unidentified weak precipitins did not contain antinuclear antibodies but two of the 4 sera with anti-K had fairly strong "homogeneous" antinuclear antibody; the serum which also contained anti-SjD had "homogeneous" titre 1/256 and that with anti-K alone had "homogeneous" titre 1/64.

DISCUSSION

Significance of Antinuclear Antibodies in Pernicious Anaemia

The incidence of these antibodies was considerably higher in pernicious anaemia (18%) than that found in the control series (6%), but the difference was not considered significant as it could have occurred by chance 1 in 10 times. If this statistical interpretation

is valid, my results would agree broadly with the only previous survey in which Doniach et al. (1963) found that the incidence of antinuclear antibodies was lower in pernicious anaemia than that in matched controls. However, the number of patients and controls which I have tested may have been too small to allow statistical demonstration of small, but valid, difference in the incidence of antinuclear antibodies between the two groups. This suggestion of a true small increase in the incidence of antinuclear antibodies in pernicious anaemia is supported by the following evidence:

(a) the higher titres of the antinuclear antibodies found in pernicious anaemia (fig. 8/27).

(b) the presence of two types of antinuclear antibody in pernicious anaemia (Table 8/35).

(c) the occasional previous finding of L. E. cells in pernicious anaemia (Berman et al., 1950).

(d) other evidence of non-tissue-specific autoimmunity in the pernicious anaemia patients in this series (see below).

A larger series of pernicious anaemia patients is being collected so that this statistical argument can be resolved.

Evidence of Systemic Autoimmunity in Pernicious Anaemia

The main manifestations of autoimmunity in pernicious anaemia are, of course, the autoantibodies to parietal cells and intrinsic factor. The picture is not, however, typically that of organ-specific autoimmunity as the patients show a disproportionately high incidence

of antithyroid antibodies.

Dr J. R. Anderson has shown that rheumatoid factor and anti-tissue precipitating antibodies were commoner in pernicious anaemia patients than in controls. Rheumatoid factor was uncommon in healthy people but frequent in "connective tissue diseases" whereas the precipitating antibodies (Anderson et al., 1961b, 1962) were very rare in the absence of systemic autoimmunity.

Even if the 3 weak unidentified precipitins are not considered, there remain 4 sera with anti-K (one with anti-SjD also) in this series of 50 pernicious anaemia patients. There is no doubt that anti-SjD is an autoantibody; the status of anti-K (whether iso- or auto-antibody) has not yet been settled, but this is probably not important in the present argument as the only previously detected precipitating iso-antibody (anti-HA) has been restricted to "connective tissue disease" patients.

The presence of precipitating anti-tissue antibodies and the raised incidence of rheumatoid factor suggested strongly that there was some degree of systemic autoimmunity in pernicious anaemia. It was probable that this is responsible for the raised incidence of antinuclear antibodies found in this survey. It therefore appeared that pernicious anaemia occupied an intermediate position between the groups of non-organ-specific and organ-specific autoimmune diseases which were separated by Hijmans et al. (1961).

SUMMARY

Antinuclear antibodies were detected in the sera of 9 of 50 patients with rheumatoid arthritis (18%) but this incidence was not significantly greater than that in controls (6%) with the numbers studied. The "homogeneous" antinuclear antibody was present in all the positive sera, one of which had antinucleolar antibody in addition. The titres of the antinuclear antibodies were low.

Evidence of some degree of systemic autoimmunity in pernicious anaemia has been presented.

H. MISCELLANEOUS SKIN DISEASES

A series of patients attending Dr N. R. Rowell's clinic (in the Dermatology Department, General Infirmary, Leeds) with diseases which showed a clinical or pathological resemblance to the "connective tissue diseases" or with rare diseases of unknown origin were tested by the fluorescent antibody technique for antinuclear antibodies. The results are shown in Table 8/37.

Antinuclear antibodies were detected in one of three patients with generalised cutaneous scleroderma and in two of nine patients with morphoea (localised cutaneous scleroderma). These three patients' sera all showed the "homogeneous" antinuclear antibody, titre 1/64 (generalised scleroderma) and 1/16 (localised scleroderma), but tests for precipitins and rheumatoid factor were negative. These tests confirm the clinical impression that these diseases should be

classified with progressive systemic sclerosis until further information is available.

In a group of five patients with nodular vasculitis, one patient had high titre (1/256) "homogeneous" antinuclear antibody. When this patient was first seen she showed no evidence of systemic involvement but 9 months later she developed systemic lupus erythematosus. Tests for rheumatoid factor and precipitating were negative initially but she later developed anti-SjD precipitin. The other four patients have not shown any systemic manifestations and tests for autoantibodies have remained negative.

None of the patients with other diseases showed antinuclear antibodies but the findings did not necessarily exclude autoimmune phenomena from these diseases as only small numbers of sera have been available for testing. The most noteworthy negative findings were:

(a) absence of antinuclear antibodies from the other arteritic diseases in view of the histological similarities to the "connective tissue diseases".

(b) absence of antinuclear antibodies from erythema multiforme in view of the characteristic immunological findings in discoid lupus erythematosus patients with erythema multiforme-like lesions.

(c) absence of antinuclear antibodies from idiopathic Raynaud's phenomenon and chilblains in view of the increased incidence when these lesions are present in association with discoid lupus

erythematosus.

SUMMARY

Antinuclear antibodies were found in 4 of a series of 103 patients with skin diseases. Three of these patients had scleroderma (one generalised cutaneous, "homogeneous" antibody titre 1/64; two localised, "homogeneous" antibody titre 1/16). The fourth patient presented as nodular vasculitis but subsequently developed systemic lupus erythematosus.

Antinuclear antibodies were not detected in other diseases which had a resemblance to the "connective tissue diseases" nor in rare diseases of unknown origin.

GENERAL DISCUSSION ON ANTINUCLEAR ANTIBODIES IN HUMAN DISEASE

Incidence, Titres and Types of Antinuclear Antibodies in Various Diseases

In general, there was a close relationship between the incidence and titres of antinuclear antibodies in various clinical syndromes. Thus, a high incidence was found in systemic lupus erythematosus (82.5%) and progressive systemic sclerosis (78%) where the titres were high, the incidence was somewhat lower in Sjögren's syndrome (62%) and discoid lupus erythematosus (35%) where the titres were in the intermediate range, whereas there was a low incidence in rheumatoid arthritis (24%) and pernicious anaemia (18%) where the titres were low. These findings have shown that the incidence and titres of antinuclear antibodies usually reflected the degree of systemic involvement in the clinical syndromes. This generalisation was most obvious when systemic lupus erythematosus was compared with the discoid form of the disease, but it was also seen when progressive systemic sclerosis was compared with generalised cutaneous scleroderma (1/3) and morphoea (2/9).

It does not hold for polyarteritis nodosa and dermatomyositis (where there is widespread systemic involvement) as antinuclear antibodies were not detected in these diseases; the numbers of patients studied were small but these findings must raise the question whether these diseases should be classified with the other "connective tissue diseases", as antinuclear antibody was detected in

only 1 of 91 patients with miscellaneous skin diseases and that patient subsequently developed systemic lupus erythematosus.

The most useful indicators of the relative frequency of different systems of antinuclear antibodies in particular diseases have been found to be the H:S ratio and the incidence of antinucleolar antibody; these have been summarised in Table 8/38. Certain similarities were apparent. The H:S ratio and the incidence of antinucleolar antibody were almost identical in the discoid and systemic forms of lupus erythematosus, although these forms were distinguished by the higher overall incidence of antinuclear antibodies in the latter group. A similar relationship (in this case a high H:S ratio and absence of antinucleolar antibody) was seen between uncomplicated rheumatoid arthritis and Group A Sjögren's syndrome (keratoconjunctivitis sicca with rheumatoid arthritis) and furthermore, the H:S ratio was high in the discoid lupus erythematosus patients who complained of joint pains (Table 8/3). There were also conspicuous differences between certain diseases. Although the H:S ratio in progressive systemic sclerosis was closely similar to that in lupus erythematosus, the diseases were distinguished by the high incidence of antinucleolar antibody in the former disease. Furthermore, high incidences of antinucleolar antibody were seen in progressive systemic sclerosis and Sjögren's syndrome (particularly Group E, the sicca syndrome alone) but the H:S ratio was considerably lower in the latter group.

As a group, the "connective tissue diseases" were characterised by a high incidence of antinuclear antibodies. These diseases had been classified together on the basis of an overlap of clinical and pathological manifestations between the individual syndromes and this grouping was supported by the present survey which has shown a similar overlap of the different systems of antinuclear antibodies. The individual syndromes of the "connective tissue diseases" had been separated because of differences in the clinical and morbid anatomical appearances; the findings recorded in this chapter have shown that in terms of the titres and relative frequencies of different systems of antinuclear antibodies, differences have also been observed between the individual "connective tissue diseases". Closely similar immunological responses have been seen in syndromes which are related clinically (e.g. the discoid and systemic forms of lupus erythematosus) whereas more marked differences were seen between less closely related syndromes.

Antinuclear antibodies were rare in patients with miscellaneous skin diseases or in random hospital patients who had not been suspected of suffering from autoimmune disease. Therefore the finding of low titre antinuclear antibodies in diseases of organ-specific autoimmunity (e.g. pernicious anaemia, p.155; Hashimoto's thyroiditis, Hijmans et al., 1961; ulcerative colitis, Calabresi et al., 1961) suggested that although release of hidden organ-specific antigens is almost certainly the major defect in these

diseases, there might also be a minor upset of immunological tolerance with consequent appearance of non-organ-specific autoimmunity in patients whose lesions were apparently the result of organ-specific autoimmunity.

The Significance of the Detection of Antinuclear Antibodies in the Individual Patient

High titre antinuclear antibodies suggest strongly that the patient is suffering from a "connective tissue disease" with systemic involvement as this finding has not been observed in other systemic diseases. Such a result has been valuable in the differential diagnosis of certain patients who had been originally suspected of suffering from subacute bacterial endocarditis and miliary tuberculosis but who were shown ultimately to be suffering from systemic lupus erythematosus.

If the fluorescent antibody test for antinuclear antibodies is negative or if low titre antinuclear antibodies are found, the result is of little use in diagnosis because:

(a) the test was negative in a small proportion of patients with the severe "connective tissue disease".

(b) it was quite commonly negative in the less severe "connective tissue diseases".

(c) it might be positive at low titre in a small proportion of patients with diseases of organ-specific autoimmunity.

(d) it was rarely positive at low titre in patients who were known

to be suffering from diseases in which autoimmunity was not known to occur.

It has been shown that antinuclear antibodies were frequently present in high titre in diseases with more extensive systemic involvement and in which the group of patients have a poor prognosis. It has not, however, been possible to draw prognostic conclusions for the individual patient from either the presence of antinuclear antibodies or their titres since these immunological abnormalities could not be correlated at least in systemic lupus erythematosus with the course of the disease and it has been my experience that dramatic changes in the clinical state of patients have occurred without any change in the immunological findings.

Furthermore, the finding of any system of antinuclear antibody in a patient has not proved of any value for distinguishing between the different syndromes of the "connective tissue diseases". In this respect, the immunological findings have been similar to the other clinical and pathological manifestations since none of them is by itself characteristic of a particular syndrome.

<u>The Clinical Value of the Fluorescent Antibody Test for Antinuclear Antibodies in the Diagnosis of the "Connective Tissue Diseases"</u>.

Many techniques have been suggested for the detection of non-tissue-specific autoantibodies in the serum of patients with "connective tissue diseases" but there has been no serious experimental comparison of the relative value of the different tests in clinical

practice. I have not attempted such an extensive investigation as the volume of work would have greatly exceeded my capacity without considerable technical assistance, but it was possible to compare my fluorescent antibody results with those of the L. E. cell test and the precipitin reaction for autoantibodies.

The fluorescent antibody test for antinuclear antibodies is undoubtedly superior to the L. E. cell test since the latter can detect only one system of antinuclear antibody (anti-nucleohistone) and moreover, takes much longer to read. The L. E. cell test is relatively insensitive but this lack of sensitivity is to some extent an advantage in practice as detection of the weaker antinuclear antibodies was shown to be of little value in differential diagnosis of human disease. It is important therefore when using the fluorescent antibody test for antinuclear antibodies to interpret the results of the test so that it will distinguish sera with stronger antibodies.

The precipitin test using homogenates of human tissue for detection of autoantibodies (Anderson et al., 1961b, 1962) has the serious disadvantages that weaker reactions may not be apparent for 5 days, its sensitivity is considerably less than that of the fluorescent antibody test for antinuclear antibodies and moreover only one antigen (DNA) has been identified chemically or definitely localised cytologically. The precipitin test has, however, certain important advantages, namely, different systems of antibodies can be separated readily, it is the only test which can detect certain

systems of autoantibodies (e.g. anti-SjD, anti-SjT and anti-Iup) whilst the identification of precipitating anti-DNA is now known to indicate a very poor prognosis for the patient. The precipitin and fluorescent antibody tests should not be considered as alternatives since each test can contribute complementary information. As the two tests are relatively simple and neither is time-consuming, it is my practice to use both in my diagnostic immunopathological service.

CHAPTER 9

TRANSPLACENTAL PASSAGE OF ANTINUCLEAR ANTIBODY

Circulating autoantibodies are a conspicuous feature of systemic lupus erythematosus, but their significance in the pathogenesis of this disease has not yet been established. Systemic lupus erythematosus is an uncommon disease in which there is lowered fertility (Madsen and Anderson, 1961) and a high foetal mortality (Ellis and Beeston, 1952; Donaldson and de Alvarez, 1962; Garsenstein et al., 1962; Hsu et al., 1962), so that an opportunity will not arise frequently to study the effects of maternal autoantibody transferred passively to a child across the placenta from a woman suffering from this disease. L. E. cells had been demonstrated in the blood of eleven infants whose mothers had systemic lupus erythematosus (Bridge and Foley, 1954; Berlyne et al., 1957; Burman and Oliver, 1958; Mijer and Olsen, 1958; Nathan and Snapper, 1958; Oudston et al., 1958; Beickert, 1961; Mahaux et al., 1962) but none of these infants developed any evidence of the disease. The L. E. cell test is not very sensitive and no attempt was made in these cases to determine the rate of destruction of the L. E. cell factor in the infant.

I have been able to study three infants born to two women suffering from systemic lupus erythematosus to determine whether the "homogeneous" antinuclear antibody can cross the placenta and if so,

to estimate the rate of destruction of the maternal antinuclear antibody in the infants. An account of the behaviour of antinuclear antibody in one of these infants has already been published (Beck and Rowell, 1963).

INVESTIGATIONS

The clinical histories of these patients are given in appendices 9/1 and 9/2. Both patients were under the care of Dr N. R. Rowell, Dermatology Department, General Infirmary, Leeds, who supplied the serum samples. Serum samples taken from both women before, during and after the pregnancies and from the three infants during the first four months were tested for antinuclear antibodies by my usual technique. These samples were also tested for rheumatoid factor, antithyroid antibodies and precipitating autoantibodies of the type described by Anderson et al. (1961). Samples of colostrum, milk and amniotic fluid were tested for antinuclear antibodies.

The susceptibility of the "homogeneous" antinuclear antibodies in the mother's and baby's sera to mercapto-ethanol was tested on serum samples obtained at childbirth (Mrs May.) or one week later (Mrs Dal.) using the methods described in appendix 9/3.

RESULTS

High titre "homogeneous" antinuclear antibody was demonstrated in serum samples taken from both women during pregnancy, at parturition and in the puerperium. The titres showed minor fluctuations (figs. 9/1, 9/2 and 9/3) but remained high throughout the period of study. Cord blood samples from the two infants born to Mrs May.

contained "homogeneous" antinuclear antibody; in one case the titre was identical to that found in the mother's blood at that time (fig. 9/2) and in the other it was one dilution step lower (fig. 9/1). The first sample was obtained from the third infant at the age of one week; this contained "homogeneous" antinuclear antibody and the titre was similar to that found in a sample of maternal serum obtained on the same day (fig. 9/3). The "homogeneous" antinuclear antibodies in the mothers' and babies' sera were resistant to treatment with mercapto-ethanol (Table 9/1).

The serial samples from each infant were titrated simultaneously in order to obtain strictly comparable titres. The findings are shown in figs. 9/1, 9/2 and 9/3. The half lives of the maternal "homogeneous" antinuclear antibodies in the three infants are 17.5, 16.5 and 16.0 days respectively.

Rheumatoid factor, antithyroid antibodies and precipitating autoantibodies were absent from both maternal and neonatal sera.

Various specimens of colostrum, milk and amniotic fluid were tested for antinuclear antibodies (see Table 9/2) but all were negative.

DISCUSSION

Placental Permeability to Antinuclear Antibody

It is now well established from direct ultracentrifugal observations on maternal and foetal cord bloods that the human placenta is freely permeable to 7.0S γ globulin but not to 19.0S

macroglobulin (Franklin and Kunkel, 1958). Furthermore, there is considerable evidence that only 7.0S γ globulin antibodies cross the placenta (see comprehensive review by Freda, 1962). As antinuclear antibodies resistant to degradation by mercapto-ethanol are probably 7.0S γ globulins (Weir and Holborow, 1962), it is not surprising that in the three pregnancies described in this chapter, the titres of "homogeneous" antinuclear antibodies were similar in mother and baby at or about the time of birth. Since the L. E. cell factor (which is always in the 7.0S fraction, Holman and Kunkel, 1957) and the "homogeneous" antinuclear antibody are probably both manifestations of anti-nucleohistone, the present observations confirm the previous reports of placental transfer of the L. E. cell factor. One of these reports (Oudsten et al., 1958) showed that although the L. E. cell factor crossed the placenta, the rheumatoid factor did not and this finding is readily explicable since the rheumatoid factor is always a high molecular weight globulin, usually 19.0S (Kunkel et al. 1959; James et al., 1961) but slight variations in the sedimentation coefficient have been recorded (Heimer et al., 1961);

Half Life of Passively Transferred Antibody

There is a constant metabolic turnover of γ globulin so that the rate of decay is logarithmic. Attempts have been made to determine the normal rate by various methods. As there is little synthesis of γ globulin in the neonate, Orlandini et al. (1955) have followed the rate of fall of the γ globulin level and estimated the

half life at 20 days. Other estimates based on the rate of decay of maternal antibodies which do not react with the infant's tissues have suggested half life values from 15 days (Mollison, 1956) to 30 days (Wiener, 1951). Wiener (1951) has emphasised, however, that the half life will be greatly reduced if the antibody reacts with an antigen in the infant's tissues, e.g. when maternal anti-A red cell isoantibody is passively transferred to a baby with blood group A.

In the three infants studied above the half life of maternal "homogeneous" antinuclear antibody was 17.5, 16.5 and 16.0 days respectively. These values, although showing individual variation are within the range of the different estimates of the half life of normal γ globulin and of antibodies which do not react with the infant's tissues. In view of Wiener's (1951) results this would indicate that the "homogeneous" antinuclear antibody is not reacting in vivo with its antigen (nucleohistone) despite the abundance of this antigen in the infant's tissue but this is hardly surprising as nucleohistone is intracellular and the cell membrane is impermeable to antibodies.

Although it is now well established that antithyroid antibodies can be transferred from mother to foetus across the placenta (Parker and Beierwaltes, 1961; Hjort and Pedersen, 1962), there has been only one report of serial investigations of the titres of these antibodies in a single infant (Mahaux et al., 1962). These authors showed that the titre of complement fixation with thyroid extracts fell fourfold in the first 19 days of life, whereas the

haemagglutination titre for antithyroglobulin fell a hundred-fold. The rapid fall in titre of antithyroglobulin might have resulted from reaction with circulating "thyroglobulin" activity which Hjort and Pedersen (1962) have demonstrated in the serum of 75% of newborn infants. There was no clinical evidence of thyroid disease in any of the infants and it seems unlikely that passively acquired anti-thyroid antibody can affect the thyroid of the recipient. Blizzard et al. (1960) have suggested that maternal antithyroid antibody may cause cretinism but subsequent investigations with experimentally induced thyroiditis in guinea pigs have failed to confirm this (Sclare and Taylor, 1961; Chandler et al., 1962) and it may be that Blizzard's clinical results reflect the familial incidence of all thyroid diseases reported by Doniach et al. (1961).

The Significance of Antinuclear Antibodies in the Pathogenesis of Disease

1. Experiments in vitro Sera containing the L. E. factor have been shown to have no effect on a variety of cell types in tissue culture (Holman et al., 1959; Lachmann, 1961b; Schoffer, 1961; Rapp, 1962b) nor on the synthesis of nucleic acids in human peripheral blood leucocytes grown in tissue culture (Williams and Schilling, 1961). The cells have undoubtedly been protected by the selective permeability of their cell membranes as Feltkamp and Kruyff (1963) have shown that antinuclear antibody can penetrate into chicken red cells only after damage to the cell membrane and Williams and Bollum (1963) have shown recently that sera containing anti-DNA cause marked inhibition of DNA-primed enzymic DNA synthesis. The other antinuclear

antibodies ("speckled" and "nucleolar") have not been investigated for cytotoxic activity.

It is well known that experimentally-produced antibodies to foreign cells have a cytotoxic effect on the corresponding cells (Wissler, 1962). It must not be assumed that human autoantibodies are necessarily incapable of producing this effect as it is now well established that certain Hashimoto's disease sera have a cytotoxic effect on human thyroid cells in tissue culture and that this activity is closely correlated with the presence of anti-microsomal antibody in the sera (Doniach and Roitt, 1962).

2. Evidence from observations in vivo Although the "homogeneous" antinuclear antibody can cross the placenta, there is very little evidence that lupus erythematosus or other "connective tissue diseases" can be transmitted in this way, since there have not been any reports of definite cases of congenital lupus erythematosus in infants born to a large number of mothers suffering from lupus erythematosus during pregnancy (Ellis and Boreston, 1952; Donaldson and de Alvarez, 1962; Garsenstein et al., 1962). The two cases claimed by Hogg (1957) and Nice (1962) respectively cannot be accepted since these authors have based their diagnosis on inadequate evidence. Epstein and Litt (1961) have reported typical discoid lupus erythematosus in a newborn infant whose mother had rheumatoid arthritis and McCuistion and Schoch (1954) have described possible congenital discoid lupus erythematosus in an infant whose mother subsequently developed

systemic lupus erythematosus but no immunological studies were undertaken in these cases. In view of the well-established evidence of familial incidence of the "connective tissue diseases"(reviewed by Peterson and Good, 1963), the occasional occurrence of these diseases in the newborn need not necessarily imply that the disease has been transmitted across the placenta.

Furthermore, transfusion of sera containing the L. E. cell factor into adult human patients did not produce any evidence of lupus erythematosus although it induced a temporary L. E. cell phenomenon (Bonsze et al., 1958 and 1959; Marmont et al., 1962). No information is available, either from pregnancy studies or from experimental transfusions, of the effects of passive transfer of other systems of antinuclear antibodies.

In contrast to these results with antinuclear antibodies, transplacental transfer of iso- and auto-antibodies to the cellular constituents of the blood can cause marked depression of the corresponding cells. Shulman et al., (1962) have demonstrated that maternal anti-platelet antibody can cause thrombocytopenia and neonatal purpura in the offspring and Lubby and Slobody (1956) and Jensen (1960) have shown that maternal anti-leucocyte antibody can cause leucopenia in the infant whilst it is well known that Rh antibodies can produce haemolytic anaemia. Thus, the antibodies to the blood cells clearly play a part in the depression of the corresponding cells but observations on passive immunisation with anti-tissue

antibodies give no indication that such antibodies can play a primary pathogenetic role in the development of the "connective tissue diseases".

Antinuclear Antibodies in Fluids other than Blood

Antinuclear antibody could not be detected by the fluorescent antibody test on 10X concentrated amniotic fluid but this result is hardly surprising as this fluid has a very low γ globulin content (Derrington and Soothill, 1961); further concentration was not possible because of the small volumes of samples available.

The absence of antinuclear antibody from both colostrum and milk was interesting as most investigations on breast secretion of anti-bacterial and antitoxic antibodies have shown antibody in colostrum only (van Genderen, 1934; Weiss, 1939; Nordbring, 1952). In the cases I have studied it is possible that the antinuclear antibody was not secreted into the colostrum specimens but it is also possible that secreted antibody was absorbed by nuclear material in the cellular debris which is always present in these specimens.

Antinuclear antibody has, however, been demonstrated in other extravascular fluids since passive L. E. tests have been shown using urine (Hauser, 1952; Korting and Schmitz, 1952; Carlson and Mollenborg, 1958), pleural fluid (van Doormaal and Schreuder, 1950; Lachmann, 1961a; Tedeschi and Nava, 1962), pericardial fluid (Seaman and Christian, 1952; Lachmann, 1961a) cerebrospinal fluid (Benczo, 1956) and joint fluid (Franke and Woerdehoff, 1951) from patients

with systemic lupus erythematosus. It seems highly probable that in these cases antinuclear antibody has passed out in the γ globulin of an inflammatory exudate as true L. E. cells have been demonstrated *in vivo*:

(a) in experimentally provoked cutaneous inflammatory lesions in systemic lupus erythematosus patients (Watson *et al.*, 1951; Perillie *et al.*, 1960) and

(b) in normal patients after local application of L. E. serum to the inflammatory site (Redbuck and Berman, 1950).

SUMMARY

Studies on 3 pregnancies in 2 women with systemic lupus erythematosus have shown that the "homogeneous" antinuclear antibody had crossed the placenta and at birth the titres in the babies' sera were similar to those in the maternal sera. In each case the antinuclear antibody was a 7.0S globulin. All 3 babies were healthy at birth and none developed any evidence of lupus erythematosus. Serial studies showed that the half life of the antinuclear antibodies were similar to those of antibodies to exogenous antigens. This suggested that the antinuclear antibodies were not reacting with their antigen *in vivo*. Antinuclear antibody could not be detected in amniotic fluid, colostrum or milk.

PART IV

(Chapters 10, 11 and 12)

CYTOLOGICAL APPLICATIONS

CHAPTER 10

THE BEHAVIOUR OF NUCLEAR ANTIGENS DURING MITOSIS

THE BEHAVIOUR OF NUCLEAR ANTIGENS DURING MITOSIS

The morphological and functional changes occurring during cell division by mitosis have been widely investigated (see reviews by Brachet, 1957 and Mazia, 1961) but conventional cytochemistry has yielded little information on the behaviour of individual proteins or on the state of combination of the various macromolecules involved. The fluorescent antibody technique could be a valuable method for tracing various nuclear antigens because of its specificity and cytological localisation. This technique is commonly used with antibodies which have been prepared in an experimental animal by injection of purified preparations of the antigen which is being studied. With this approach the technique will be limited to the study of those antigens which can be effectively purified and which are also antigenic to the experimental animal used for the production of the immune serum. Unfortunately, antibodies cannot be prepared against substances, such as nucleohistone, to which experimental animals are immunologically tolerant. This chapter describes the application of the various types of autoimmune antinuclear antibodies (described in Chapters 2, 3 and 4) to trace their corresponding antigens in HeLa cells in various stages of mitosis. These human autoimmune antinuclear antibodies have proved valuable reagents for investigation of mitosis because

(a) they were already characterised

(b) they were available in high titre sera

(c) they reacted with nuclear constituents which are not ordinarily antigenic.

MATERIALS AND METHODS

(a) Tissue Culture HeLa cells were grown on coverslips in tissue culture in a modified Hank's medium with the following composition -

Hank's medium	80%
Bovine serum	10%
Lactalbumin	0.25%
Hartley's broth	5%
1.4% Sodium bicarbonate	4%

(b) Human Sera

(i) "Homogeneous" sera The sera used were obtained from five patients, Dun., War., Mot., Bry. and Pea.

(ii) "Speckled" sera Sera were obtained from five patients, Tra., McDon., McLar., Pat. and Nis.

(iii) "Nucleolar" sera No patient has yet been encountered whose serum contains this antibody alone. Two patients' sera have been used. The first from patient Mui. contained a high titre antinucleolar antibody with low titre anti-nucleohistone. This serum was used after the anti-nucleohistone had been removed by absorption with calf thymus nucleohistone. The second serum from patient Ser. contained antinucleolar and "speckled" antibody. This has been used both without absorption and after absorption with a preparation of saline-soluble nuclear proteins.

(c) Staining technique The coverslip preparations were washed in barbitone-buffered saline (pH 7.2) for 5 min. with gentle agitation

to remove the culture medium. Fixation of the tissue was necessary to allow the antibodies to penetrate into the cells, but the conditions were critical, since the antigens are rather labile. Fairly satisfactory and reproducible results were obtained by immersion of the moist coverslip preparation in "Reagent grade" acetone at room temperature for 5 sec. The preparation was then immediately transferred to barbitone-buffered 0.15M-NaCl.

Fixed coverslip preparations were treated with diluted patient's serum in a moist chamber for 30 min. at room temperature, washed for 10 min. in buffered saline and then treated for 30 min. with fluorescein-conjugated rabbit anti-human-γ-globulin (see appendix 1/1). These preparations were then washed for 10 min. in buffered saline with gentle agitation, mounted in buffered glycerol (pH 7.2) and finally sealed to the slide with nail varnish. Care had to be taken to avoid contact of nail varnish with the tissue culture as the solvent quenches the fluorescence of the fluorescein. The preparations were examined with dark ground blue-violet/ultraviolet light and photographed on high speed Ektochrome.

The specificity of the fluorescein-conjugated anti-human-γ-globulin was established by the following controls:

(i) No staining was produced if the conjugated anti-human-γ-globulin had been absorbed with normal human γ globulin prepared from human serum on a DEAE cellulose column.

(ii) Pre-treatment with unconjugated rabbit anti-human-γ-

globulin prevented staining by the conjugated antibody.

(iii) No staining was produced when fluorescein-conjugated anti-human serum albumin was substituted for fluorescein-conjugated anti-human-γ-globulin.

(d) Enzyme digestion of antigens Fixed coverslip preparations were incubated for 1 hr at 37°C in a moist chamber with either Worthington deoxyribonuclease (DNase) (0.1mg./ml.) in barbitone-buffered 0.15M-NaCl (pH 7.2) containing 40 µg.Mg ++/ml. or with Worthington ribonuclease (RNase) (1.0 mg./ml.) in ion-exchange water. Control preparations were incubated with pH 7.2 barbitone-buffered saline containing 40 µg.Mg ++/ml. or with ion-exchange water.

(e) Absorption of antibodies Nucleohistone was isolated from calf thymus by the low ionic strength method (Chargaff, 1955). Various patients' sera were absorbed with this preparation using approximately 25 mg./ml. of serum by incubation at 37°C for 45 min.; after centrifugation, the supernatant was used to stain coverslip preparations. Absorptions were also attempted with saline-soluble nuclear proteins prepared as described in Chapter 3.

RESULTS

The term "homogeneous" and "speckled" were applied in Chapter 1 to describe the appearances of nuclei stained by two different types of antinuclear antibodies in unfixed sections of snap frozen liver. Anti-nucleohistone causes overall ("homogeneous") staining (fig. 1/1) whereas the antibody reacting with the saline-soluble component of

nuclei causes finely speckled staining (fig. 1/2) under these conditions. Since the antinuclear antibody cannot penetrate intact cell membranes, the patients' sera cannot be used to stain unfixed HeLa cell tissue cultures. The acetone treatment used to destroy the selective permeability of the cell membrane resulted in variable alteration in the physico-chemical state of the nuclear constituents so that the distinctive patterns of nuclear staining are no longer produced. The terms, "homogeneous" and "speckled", although not strictly valid in descriptions of tissue cultures will nevertheless be retained to avoid unnecessary confusion by the introduction of a new terminology.

1. Results obtained with "Homogeneous" Antinuclear Antibody

The earliest change of prophase which was recognised, was the emergence of strands within the nucleus (fig. 10/1). These strands progressively shortened and became prominent (fig. 10/2), and at a later stage the intensely stained chromosomes became separated. In such preparations, each chromosome appeared uniformly stained throughout its length and thickness (fig. 10/3).

Cells in metaphase show intense specific staining of the chromosomal plate, whereas the cytoplasm was completely unstained (fig. 10/8). The chromosomes were now so closely packed that the metaphase plate was stained as a uniform mass but the centrioles and the spindle were unstained. During anaphase, the separating chromosomal plates were stained as a uniform mass (fig. 10/11 and

10/12) whereas the cytoplasm remained unstained. Later, in early telophase, there was intense specific staining of the chromosomes which were separating from the chromosomal plate (fig. 10/16). After the nuclear membranes developed round the daughter nuclei, ill-defined stranding was noted in the nuclei (fig. 10/17). At a later stage, the daughter nuclei reverted to the interphase pattern.

If fixed tissue cultures were incubated with DNase before the staining procedure, interphase nuclei and chromosomes were completely unstained. Incubation with the control buffer or treatment with RNase did not alter the pattern of staining of interphase nuclei and chromosomes produced by these antisera.

Absorption of the sera with calf thymus nucleohistone abolished the staining of interphase nuclei and chromosomes, whereas absorption with the saline extract of isolated nuclei had no effect on the staining properties of these sera.

2. Results obtained with "Speckled" Antinuclear Antibodies In the earliest stage of prophase which was recognised, the nucleus showed a reticulated pattern of staining on which the unstained areas corresponded to the separating chromosomes, while the cytoplasm also showed intense staining (fig. 10/4). In late prophase, the antigen was restricted to the cytoplasm and formed a halo round the unstained nucleus. At this stage, the "speckled" antigen was most concentrated in the perinuclear region of the cytoplasm (fig. 10/5). In metaphase, the "speckled" antigen was usually restricted to the

cytoplasm, with highest concentration adjacent to the chromosomal plate. However, thin strands were sometimes seen penetrating across the chromosomal plate (fig. 10/9). These strands probably represent the spaces between the individual chromosomes.

The chromosomal plates separating in anaphase showed a similar appearance to chromosomal plates in metaphase, whilst the antigen was present mainly in the poles of the cells, but its distribution did not correspond to that of the spindle (fig. 10/13). In later anaphase, the antigen formed a fairly uniform halo round the chromosomal plates, which were usually unstained (fig. 10/14). When the daughter nuclei were undergoing reconstruction in telophase, the antigen was found both in the nuclei and in the surrounding cytoplasm (fig. 10/18) but finally the antigen was restricted to the new interphase daughter nuclei.

Pretreatment of the fixed smears with either DNase or RNase did not affect the appearances produced by fluorescent antibody staining using the "speckled" antinuclear sera.

Absorption of the "speckled" sera with a saline extract of isolated rat liver nuclei prevented the staining patterns described above whereas absorption with calf thymus nucleohistone did not affect the staining properties of these sera.

3. Results obtained with "Nucleolar" Antinuclear Antibody In early prophase the nucleoli were seen adhering to the separating chromosomes (fig. 10/6) but in later prophase, the nucleolar antigen has

passed into the cytoplasm around and between the separating chromosomes (fig. 10/7). In metaphase, the nucleolar antigen was restricted to the cytoplasm while the central area of the chromosomal plate is unstained (fig. 10/10). In this photograph, the irregular brightness in the centre of the chromosomal plate area was pale blue autofluorescence in contrast to the yellow-green specific staining of the antigen in the cytoplasm. In anaphase, the separating chromosomal plates were unstained and the antigen was located in the surrounding cytoplasm (fig. 10/15). In late telophase, the antigen reappeared in the reforming nucleoli.

Pretreatment of the fixed smears with either DNase or RNase did not affect the appearances produced by fluorescent antibody staining using the antinucleolar sera. The antinucleolar antibody was not absorbed by treatment with either calf thymus nucleohistone or saline extract of nuclei.

DISCUSSION

"Homogeneous" Antinuclear Antibody It has been shown that sera of this type contain antibodies to nucleohistone and the results presented above demonstrated that the staining patterns produced by such sera corresponded to the classical appearances of the chromosomes in mitosis (Fell and Hughes, 1949). It can therefore be deduced that the nucleohistone antigen is a constituent of the chromosomes of HeLa cells. This confirmed the findings of Kroath et al. (1961) that certain human sera containing antinuclear antibodies

stained separated mammalian metaphase chromosomes throughout their substance. They did not identify the antigen with which these sera reacted, but in view of my experiments, it seems highly probable that the sera contained anti-nucleohistone. Furthermore, they obtained chromosomal staining with the sera of 5 of 6 patients with lupus erythematosus, a disease in which antinucleohistone is frequently a prominent feature (Table 8/38).

Rapp (1962a and b) has also demonstrated staining of separated metaphase chromosomes by sera containing antinuclear antibodies of unspecified type but he illustrated other preparations of intact cells where the chromosomes are stained at their tips only. It is difficult to resolve this inconsistency but it is possible that the nucleohistone in the intact cells had become hydrophobic as the result of air drying of his preparations.

The distribution of antigenically identifiable nucleohistone in cells at various stages of mitosis corresponded to the distribution of deoxyribonucleic acid (DNA) shown by Feulgen staining (Jacobson and Webb, 1952) and by ultraviolet absorption micrography at 2600 Å (Davies, 1952). Cytochemical observations on the giant salivary chromosomes of Diptera had suggested that DNA and histone are usually found together (Serra and Queiroz-Lopez, 1944; Caspersson, 1950) and this has been confirmed by Mazia (1950) by removal of non-histone residual protein by digestion with a highly purified pepsin preparation to which histone is resistant. Recent observations

with the interference microscope suggest that the DNA-histone content of metaphase chromosomes may be as high as 85% (Mellors, 1955). These cytochemical techniques cannot establish whether DNA and histone are free or combined, although by careful analysis of May-Grünwald and Geimsa staining, Jacobson and Webb (1952) concluded rather empirically that DNA was combined as nucleohistone in chromosomes. The new methods used in this investigation have shown conclusively by an immunological method that DNA is combined with histone as nucleohistone in both interphase nuclei and chromosomes. This confirms the assertion of Anderson and Fisher (1961) and Beck (1962b) that nucleohistone is a true constituent of interphase nuclei, whilst the demonstration of nucleohistone in chromosomes supports the claim that nucleohistone strands in interphase nuclei represents the fundamental units of chromosome structure (Anderson and Fisher, 1961).

"Speckled" Antinuclear Antibody It was shown in Chapter 3 that sera of this type contain an antibody to a saline-soluble nuclear protein. The observations reported above showed that although this antigen was fairly uniformly distributed within interphase nuclei, it was not a constituent of chromosomes but was transferred into the surrounding cytoplasm during prophase and returned to the nucleus in telophase. This would be compatible with the observation that certain human sera containing antinuclear antibodies of unspecified type (from 1 of 6 patients with lupus erythematosus and all of 3

patients with Sjögren's syndrome) did not stain isolated metaphase chromosomes (Krooth et al., 1961) as the "speckled" antibody is commonly present in high titre in Sjögren's syndrome whilst it is less common to find this antibody as the predominant antibody in lupus erythematosus (Table 8/38).

This immunocytochemical demonstration of the transfer of the "speckled" antigen into the adjacent cytoplasm during mitosis is of interest since it helps to explain interferometric observations of the loss of dry mass from the nuclear apparatus with weight gain in the adjacent cytoplasm during division of both plant and animal cells (Richards, 1960; Ambrose and Bajer, 1961; Richard and Bajer, 1961). In plant cells, Richards (1960) and Ambrose and Bajer (1961) describe a peri-chromosomal pale-staining zone where mass gain is greatest and similar pale-staining areas have been shown around the chromosomal plates in HeLa cells in tissue culture (Hsu, 1954) and in human epidermis in vivo (Schering, 1959). These "clear" areas correspond closely to the distribution of the "speckled" antigen at metaphase. It thus seems highly probable that part of the loss of nuclear mass during prophase can be explained by the transfer of the "speckled" antigen to the cytoplasm.

It is interesting to speculate on the mechanisms underlying the dissociation of the "speckled" antigen from the chromosomes during mitosis. The two most likely hypotheses would seem to be:

(a) the "speckled" antigen is normally held in the interphase

nucleus by the nuclear membrane or

(b) the physical properties of nucleohistone become altered when the chromosomes separate in mitosis so that the "speckled" antigen is no longer firmly bound.

The first suggestion seems unlikely since the "speckled" antigen starts to leave the nucleus in prophase while the nuclear membrane is still intact. Moreover, recent studies suggest that the nuclear membrane of isolated nuclei is permeable to nucleases (Anderson, 1953), haemoglobin (Holtfreter, 1954), soluble nuclear proteins (Barton, 1960) and human 7.0S γ globulin (Beck, 1962a). The validity of these observations could be questioned on the ground that isolated nuclei might not behave in a physiological manner, but Allfrey et al. (1957) have shown that nuclei isolated in sucrose retain their capacity for protein synthesis and similar, presumably viable, nuclei have been used in some of my experiments.

The results of in vivo experiments are less clear cut as Fischer and Wagner (1954) have shown that the nuclei of intact cells are freely permeable to protamine and histone whereas Harding and Feldherr (1958, 1959) claim that the nuclear membrane is impermeable to bovine serum albumin and polyvinylpyrrolidine. Thus, although the nuclear membrane may show some selective permeability to macromolecules, Barton's (1960) results suggest that the nuclear membrane is not the main factor retaining the "speckled" antigen in the interphase nucleus.

The second hypothesis would seem more likely if Barton's (1960)

suggestion that in interphase nuclei nucleohistone holds soluble nuclear proteins by ionic linkages, should prove to be valid. During prophase the nucleohistone undergoes condensation while ribonucleic acid (Brachet, 1940; Kaufmann et al., 1948; Jacobson and Webb, 1952; Ris and Kleinfeld, 1952; Boss, 1955; Love, 1957; Feinendegen and Bond, 1963), zinc (Fujii, 1955) and phospholipid (La Cour et al., 1958) become incorporated in the chromosomes.

In view of changes in the physical state of the nucleohistone, it would not be surprising if the protein-binding properties of the chromosomes were different from those of the nucleohistone of the interphase nucleus so that the "speckled" antigen was released into the surrounding cytoplasm.

Antinucleolar Antibody The nature of the nucleolar antigen traced in these experiments has not yet been determined. It is of interest, however, that this nucleolar antigen does not take part in the formation of chromosomes but passes into the cytoplasm during mitosis and in this respect its behaviour is analogous to that of the silver-reducing component of nucleoli described by Das and Alfert (1959) and Tandler (1959). These results show that there is at least one component of the nucleolus which does not take part in the formation of chromosomes.

The behaviour of other constituents of the nucleolus during mitosis has not been decided conclusively. Jacobson and Webb (1952) have suggested that some of the nucleolar RNA is incorporated in the

metaphase chromosomes and this is supported by the observation (Swift et al., 1956) that the chromosomal RNA content increases when the nucleolus breaks down but the nucleolar RNA cannot account quantitatively for all the chromosomal RNA. There is some evidence that nucleolar RNA is transferred to the chromosomes when the nucleolus disappears (Godward, 1953; Woods and Taylor, 1959; Taylor, 1960; La Cour, 1963) but Feinendegen and Bond (1963) have shown conclusively that much of the chromosomal RNA is newly synthesised and not derived from the nucleolus. There is, however, also considerable evidence that some of the nucleolar RNA is transferred to the cytoplasm in metaphase (Love, 1957; Love and Suskind, 1961; Prescott and Bender, 1962). There is evidence, reviewed by Sirlin (1960) that the nucleolus contains RNA in at least two forms, structural and soluble, and these varieties of RNA may behave in different ways during cell division. The nucleolus cannot be considered, therefore, as a single entity and any description of mitosis should consider the behaviour of its different constituents separately. Succinic dehydrogenase has recently been described in nucleoli of interphase cells (De et al., 1961, 1962) but it has not been studied through mitosis; it will be interesting to hear its fate.

Interferometric observations show that although the weights of the nucleoli of the daughter cells are unequal, their combined mass is equal to the prophase nucleolus (Martin, 1961) and autoradiographic

experiments suggest that the nucleoli are reformed in telophase by aggregation of protein synthesised before mitosis (Harris, 1961). These biophysical and metabolic observations suggest that nucleoli of some cells survive cell division, but Sirlin (1961) presents morphological evidence that nucleoli may be newly synthesised in the telophase nuclei of certain other specialised cells.

The passage of the nucleolar antigen into the cytoplasm along with the "speckled" antigen will account for at least part of the loss of nuclear mass in mitosis reported by Richards (1960), Richard and Bajer (1961) and Ambrose and Bajer (1961).

Cytoplasmic division in mitosis, unlike chromosomal separation is not necessarily quantitatively equal, so that the differences in dry mass of the nucleoli of the daughter cells reported by Martin (1961) is hardly surprising.

SUMMARY

Certain monospecific human antinuclear sera were used to trace the corresponding nuclear antigens in HeLa cells at various stages of mitosis. DNA, either free ("membranous" antigen) or combined with histone ("homogeneous" antigen) condensed in prophase and was incorporated completely into the chromosomes until it was diffused in the reforming nuclei of daughter cells. The "speckled" antigen left the nucleus in early prophase and was dispersed within the cytoplasm until late telophase when it re-entered the daughter nuclei. The "nucleolar" antigen was concentrated in discrete nucleoli in

early prophase, it passed into the cytoplasm before the onset of metaphase and ultimately returned to the nucleoli which were reforming in late telophase. These experiments demonstrated that at least two of the constituents of interphase nuclei were absent from the chromosomes and so were probably not important for inheritance. Furthermore, these results would explain the previous observations of loss of nuclear mass in prophase with recovery in telophase.

CHAPTER 11

NUCLEAR ANTIGENS IN NORMAL AND LEUKAEMIC LEUCOCYTES

The morphology and cytochemistry of normal and leukaemic leucocytes have been investigated extensively by conventional histochemical techniques (Valentine, 1960) and much is known of the leucocyte cytoplasmic antigens (Killmann, 1960; Walford, 1960).

In contrast, the antigens in leucocyte nuclei have been neglected. There has been only one study with experimentally induced antibodies; in this an extract of isolated human leukaemic leucocyte nuclei was used as an antigen and the resultant immune serum was said to produce five precipitin bands when tested by immunoelectrophoresis (Atchley, 1960). This author has claimed to have identified the antigens responsible for two of these bands as histone and nucleohistone. This approach has two important disadvantages - it is not possible to determine the incidence and cytological localisation of antigens in particular cell types and immunological tolerance will limit the number of antigens which can be studied.

In many respects, human autoimmune antinuclear antibodies are more satisfactory reagents since it is possible to select high titre sera against antigens to which normal animals show immunological tolerance. This has, in fact been done, unwittingly, during previous investigations on the L. E. cell phenomenon (see introduction) since

these experiments have shown that the nuclear antigen (nucleohistone) was present in the nuclei of all the normal and leukaemic leucocytes which were studied.

Atchley (1961c) has repeated his immunoelectrophoretic experiments using lupus erythematosus serum and demonstrated nucleohistone and free histone in extracts of leukaemic leucocyte nuclei. The fluorescent antibody technique must, however, be the method of choice, since it allows cytological localisation of the antigen in individual cell types.

Rather surprisingly, although blood films have been used in many investigations as substrates in fluorescent antibody tests for antinuclear antibodies, there has been only one previous attempt to determine the distribution of nuclear antigens in different types of human leucocytes using human antinuclear sera with the fluorescent antibody technique (Gökcen, 1962). Since Gokcen's experiments were inadequately controlled and since his results appeared improbable, I decided to investigate the distribution of antigens in the nuclei of normal and leukaemic leucocytes by the fluorescent antibody technique using selected human antinuclear sera containing characterised antinuclear antibodies of the four types described in this thesis.

MATERIALS AND METHODS

Leucocyte Preparations

Studies were made on the peripheral blood of four normal subjects, four myeloid leukaemia patients and four lymphatic

leukaemia patients. Air-dried blood films were made directly from a finger prick for this investigation since I found in preliminary experiments that anticoagulants (oxalate, citrate, versene or heparin) interfered with the staining effect of human antinuclear sera on leucocyte nuclei. These preparations were tested without fixation; they could be stored at room temperature for periods up to three days without apparent deterioration of antigenicity. Several attempts were made to stain marrow cells but these were not followed up because of the difficulty in identification of cell types in these preparations.

Antinuclear Sera

The human sera used in this investigation had (with one exception, serum Pac.) been characterised previously. "Homogeneous" sera were obtained from patients Dun., Bra. and Gra. "Membranous" sera were obtained from patients Tan and Nob. "Speckled" sera were obtained from Tra., Ral., Abr. and McD. "Nucleolar" sera. All sera containing antinucleolar antibody also contained other antinuclear antibodies. For this investigation, three sera with high titre antinucleolar antibody were chosen; two of them (Mui. and Pac.) contained low titre "homogeneous" and the other (Scr.) had low titre "speckled" antinuclear antibody. These sera were used at a dilution greater than the titre of the "homogeneous" and "speckled" antinuclear antibody.

Staining Technique

Unfixed blood films were treated with antinuclear sera for 30

min., washed for 10 min. in barbitone-buffered saline (pH 7.2) and stained with fluorescein-conjugated anti-human-γ globulin for 30 min. After washing for 10 min., the preparations were mounted in buffered glycerol and examined in the usual ultraviolet optical system. Leitz "non-fluorescent immersion oil" was used with the 2 mm. objective.

In preliminary experiments, I noted that the titres of anti-nuclear sera were much lower when tested against human leucocytes in blood films than in titrations against cryostat sections of rat liver. In order to obtain sufficiently bright staining, the selected anti-nuclear sera were usually used at 1/4 dilution in the experiments reported below.

Immunological Control of the Specificity of Staining

(a) The antinuclear sera used in this investigation had been characterised previously and the specificity of their reaction was confirmed as follows:

"Homogeneous" Antigen destroyed by DNase: antibody was absorbed by nucleohistone but not by DNA.

"Membranous" Antibody destroyed by DNase: antibody was absorbed by nucleohistone and DNA.

"Speckled" Antigen removed by treatment with 0.15M-NaCl.

(b) The specificity of staining by fluorescein conjugated anti-human-γ- globulin was controlled as described on p. 182.

Identification of Cell Types

The most satisfactory method would be to restain a fluorescent

antibody preparation with a conventional dye (such as toluidine blue) so that the appearances of any cell under ultraviolet illumination could be compared with those seen under familiar conditions. This did not, however, prove successful as individual cells could not be relocated in the absence of tissue architectural features even when the mechanical stage had been locked.

The recognition of cell types was thereafter based on appearances seen with dark ground illumination and depended mainly on cell size, nucleo-cytoplasmic ratio and morphology of nuclei. The reliability of these observations was checked by comparison of differential counts, on blood films made from the same finger prick but stained separately by fluorescent antibody and the Leishman method (Tables 11/1 to 11/4).

RESULTS

Normal leucocytes All leucocyte nuclei were stained by "homogeneous" and "membranous" antinuclear sera (fig. 11/1) but it was not possible to distinguish between the staining patterns produced in leucocytes by these types of sera since both usually gave staining of similar intensity over the whole of the nucleus and "homogeneous" sera sometimes produced marked accentuation of the outlines of the nuclei.

"Speckled" antinuclear sera gave typical speckled staining in the nuclei of lymphocytes (fig. 11/2) and monocytes (fig. 11/3) but failed to stain polymorph nuclei (fig. 11/4) (neutrophil, eosinophil and basophil leucocytes could not be confidently distinguished).

Antinucleolar sera stained nucleoli in most lymphocytes (fig. 11/5) and all monocytes (fig. 11/6). There was usually one nucleolus (diameter 1 - 2 μ) in each lymphocyte nucleus and two or three nucleoli (diameter 1 - 3 μ) in monocyte nuclei. The nucleoli were rounded, usually scattered eccentrically but rarely appeared at the edge of the nucleus. Antinucleolar sera did not stain polymorph nuclei (fig. 11/7).

No difficulty was experienced in identifying cell types and the validity of my observations were confirmed by comparison of differential counts on preparations stained by the Leishman method and the "speckled" antinuclear antibody (Table 11/1).

Chronic Myeloid Leukaemia "Homogeneous" and "membranous" antinuclear sera stained the nuclei of all leucocytes but, as with normal blood, the staining patterns were indistinguishable (fig. 11/8).

The "speckled" antigen was present in the nuclei of all the primitive cells of the myeloid series (fig. 11/9). The myelocyte nuclei were brightly stained but the staining intensity was progressively less in cells showing further stages of differentiation. Moreover, in a proportion of the apparently mature polymorphs "speckled" staining was seen scattered throughout all the lobes or restricted to one lobe (fig. 11/10) but the nuclei of some of the polymorph forms were unstained as in normal blood. The proportion of polymorphs showing "speckled" staining varies in blood films from different patients and is higher in those where the polymorph nuclei

show less severe chromatin clumping in Leishman-stained preparations (Tables 11/2, 11/3, 11/4 and 11/5).

Using antinucleolar sera, nucleoli were demonstrated in the primitive cells of the myeloid series (figs. 11/11 and 11/12). Although the modal number of nucleoli in myelocytes varied in individual patients (figs. 11/13, 11/14, 11/15 and 11/16) all the patients studied showed a progressive decrease in size and number of nucleoli with increasing differentiation of the cells. There was more variation in the size and shape of the nucleoli in chronic myeloid leukaemia than in chronic lymphatic leukaemia but the eccentric position of nucleoli in the nucleus was similar to that seen in other leucocytes.

Chronic Lymphatic Leukaemia The nuclei of all abnormal cells in blood films of chronic lymphatic leukaemia were stained by "homogeneous" (fig. 11/17) "speckled" and "membranous" antinuclear sera.

Nucleoli were demonstrated by antinucleolar sera in most of the abnormal cells and their appearances are similar to those seen in normal lymphocytes (fig. 11/18). The modal number of nucleoli in the abnormal cells was 1 and the scatter (figs. 11/19 and 11/20) was similar to that seen in normal lymphocytes (figs. 11/21 and 11/22).

DISCUSSION

Nuclear Antigens in Normal Blood

"Homogeneous" and "Membranous" antinuclear sera stained the nuclei of all leucocytes. This showed that these antibodies can

penetrate into the cells in air-dried blood films. It also demonstrated that nucleohistone and DNA antigens were present in all leucocyte nuclei, but this was not surprising since these antigens have been demonstrated in the nuclei of all vertebrate cells (except spermatozoa) which I have tested.

These findings are completely different from those reported by Gökcen (1962) who claims to have identified separate antibodies to polymorph DNA and lymphocyte DNA, and that neither of these antibodies could stain normal blast cells nor leukaemic cells. It is difficult to accept these results, because:

(a) I found that all sera gave nuclear fluorescence when I used Gökcen's staining technique. There was a minor difference in staining intensity between normal and pathological sera, but this cannot be considered a satisfactory experimental method for detection of antinuclear antibodies.

(b) The specificity of action of his fluorescent anti-human γ globulin antiserum was demonstrated by a blocking test with the unconjugated antiserum. This was not an adequate control because at best it merely demonstrated that the same antibodies were present in the labelled and unlabelled antiserum. It did not prove that staining was produced by fluorescent anti-human-γ globulin.

(c) The antinuclear antibodies were assumed to be anti-DNA because the antigens were destroyed by DNase. The possibility of a nucleohistone antigen was not considered.

(d) Gökcen claimed that antinuclear sera did not stain normal or leukaemic blast cells. This was not confirmed by the work here reported.

The "speckled" antigen was present in lymphocytes and monocytes but it was absent from polymorph nuclei where the chromatin was densely clumped. Hale (1963) has shown that polymorph nuclei contain 10% less DNA than the other somatic nuclei and he has attributed this to early pyknosis. It is possible that clumped chromatin might have lost its capacity to absorb the "speckled" antigen and so retain it within the nucleus (c.f. the changes during the separation of the chromosomes in the prophase of mitosis). Pyknosis may therefore be responsible for the absence of the "speckled" antigen from polymorph nuclei in addition to the depletion of the DNA content of these cells. The distribution of the speckled antigen in normal leucocytes has not been investigated previously.

Antinucleolar antibody demonstrated one or two nucleoli in most normal lymphocytes and one to three nucleoli in almost all normal monocytes. The appearance, size and number of nucleoli in lymphocytes and monocytes are similar to those described by Smetana (1961) on preparations stained rather empirically with toluidine blue. The absence of the nucleolar antigen from polymorph nuclei confirms the previous failure to demonstrate nucleoli in the nuclei of these cells with conventional light microscopic methods (Smetana, 1960) or electron microscopy (Ham and Leeman, 1961). The distribution of

the nucleolar antigen in normal leucocytes has not been investigated previously.

Chronic Myeloid Leukaemia There was no doubt that the "homogeneous" and "membranous" antigens were present in large amounts in all leucocyte nuclei in chronic myeloid leukaemia. This disproves Gökcen's (1962) assertion that the nuclei of blast cells and other leukaemic cells did not react with antinuclear antibodies.

There was progressive loss of the "speckled" antigen with increasing differentiation of the cells of the myeloid series. This was not uniform throughout the nucleus and the antigen persisted in areas where the nucleoprotein was less densely clumped so that polymorph forms were sometimes seen with staining of only one lobe of the nucleus. The nuclei of a proportion of leukaemic polymorphs behaved like normal cells and did not contain the "speckled" antigen. Clumping of chromatin in leukaemic cells resulted in loss of affinity for the "speckled" antigen in the same manner as in normal leucopoiesis. The distribution of this antigen in myeloid leukaemia cells has not been investigated previously.

Antinucleolar antibodies have proved very sensitive staining reagents since they demonstrated two or three large and several small nucleoli in all myelocytes, several smaller nucleoli in most metamyelocytes and one or two very small nucleoli in some stab cells, whereas conventional staining methods cannot be used to demonstrate nucleoli beyond the myelo-/cyte stage where two or three fairly large

nucleoli are seen (White, 1947; Wintrobe, 1951). This difference must be attributed to the selective staining of nucleoli which was possible with the fluorescent antibody technique and the advantage of dark field illumination in which staining contrasted with the dark background of the rest of the nucleus. The small nucleoli, which would be missed with conventional staining, were readily seen by the fluorescent antibody technique so that the larger number of nucleoli demonstrated in myelocytes in this investigation is readily explained. There have not been any previous attempts to demonstrate the "nucleolar" antigen in myeloid leukaemia leucocytes.

Chronic Lymphatic Leukaemia The antigens in lymphatic leukaemic nuclei have not been investigated previously. I have shown that the "homogeneous", "membranous" and "speckled" antigens are present in chronic lymphatic leukaemia lymphocyte nuclei and that the staining patterns are indistinguishable from those seen in normal lymphocytes.

Staining with antinucleolar antibody showed that the mean number and size of nucleoli in lymphatic leukaemia cells is similar to that of normal lymphocytes. There is some evidence (reviewed by Busch et al., 1963) that the number and size of nucleoli demonstrated by conventional stains may be a little larger than in the normal lymphocytes but this was not confirmed in the present investigation.

Leucocytes as substrates for test for antinuclear antibody

Normal blood films have been used extensively by other workers as the substrate for fluorescent antibody tests for these antibodies

on the assumption that leucocyte nuclei contain all nuclear antigens. This chapter has shown that although this assumption is valid for lymphocytes and monocytes, it is not true for polymorphs. Furthermore, blood film substrates have much lower sensitivity than tissue sections, due presumably to the greater ease with which the antibody can diffuse into sectioned cells. Blood films must therefore be considered inferior to tissue sections in all respects other than the ease of preparation, so that leucocytes cannot be recommended as substrates in routine fluorescent antibody tests for antinuclear antibodies.

SUMMARY

Histochemical observations have been made on normal and leukaemic leucocytes using characterised human antinuclear sera. The "homogeneous" and "membranous" antigens (nucleohistone and DNA) respectively have been detected in all leucocyte nuclei.

The "speckled" antigen (a soluble protein in the nucleus) was absent from all normal and a proportion of leukaemia polymorphonuclear leucocytes but was present in all other types of leucocyte.

The "nucleolar" antigen was absent from all normal and leukaemic polymorphs, many leukaemic stab cells and a small proportion of normal lymphocytes but was detected in all other leucocytes. Antinucleolar antibodies have proved sensitive and specific reagents for detection of nucleoli in leucocytes.

Blood films cannot be recommended as substrates in fluorescent

antibody tests for antinuclear antibodies for three reasons:

(a) the system was much less sensitive than the rat liver test system.

(b) two of the antigens were absent from polymorphonuclear leucocytes.

(c) nuclear staining pattern was less easily recognised in leucocyte nuclei than in the larger rat liver cell nuclei.

CHAPTER 12

ANTIGENS IN THE NUCLEI OF CERTAIN PROTOZOA

Species-specificity is a prominent characteristic of experimentally induced antibodies. Although it is much less marked with the human autoimmune antinuclear antibodies, species-specificity is nevertheless not entirely lacking in these antibodies since "speckled" antinuclear antibodies will react only with mammalian nuclei (Table 3/1) and antinucleolar antibodies can be demonstrated only with vertebrate tissue (Table 4/1). The "homogeneous" and "membranous" antinuclear antibodies react with cell nuclei from a much wider range of taxonomic groups (Table 2/1 and 5/1) but preliminary experiments indicated differences between the two systems in their reaction with the nuclei of certain trypanosomes. This chapter describes observations on the distribution of the "homogeneous" and "membranous" antigens (nucleohistone and DNA respectively) in certain species of protozoa.

MATERIALS AND METHODS

Preparation of Protozoa

All organisms were tested as air-dried smears on chemically clean microscope slides. They were not fixed chemically. Various species of trypanosomes (listed in Table 12/1) (either in blood films from infected laboratory animals or from in vitro cultures) and cultures of Leishmania donovani and Strigomonas Sp. were supplied by

Dr P.J. Walker (Division of Parasitology, National Institute for Medical Research, London), Toxoplasma gondii in smears of the peritoneal exudate of infected mice was given by Dr S. Fletcher (Pathology Department, The University and Western Infirmary, Glasgow). Amoeba proteus, Peranema sp. and Paramecium sp. were obtained from the stock cultures in the Zoology Department, The University of Glasgow.

Immunological Staining Technique

The indirect method was used. The staining periods with human antinuclear sera and fluorescent anti-human-γ globulin were standardised at 30 min. and the preparations were washed for 10 min. periods in buffered saline.

Four human antinuclear sera were selected for this investigation as they were high titre and monospecific and had been characterised previously; two (Dun. and War.) contained the "homogeneous" antibody and two (McDou. and Tan.) the "membranous" antibody. Control smears were treated with two normal human sera (And. and Bec.). All sera were used at 1:4 dilution in barbitone buffered saline. The specificity of the antinuclear sera was confirmed by DNA, RNA and nucleohistone absorptions and also by prior digestion of the antigens by DNase and RNase using the methods described on p. 183. The specificity of staining by the fluorescein-conjugated anti-human-γ globulin was controlled as described on p. 199.

RESULTS

The observations on nuclear staining are summarised in Table 12/1,

which shows that the nuclei of all the protozoa were stained by sera containing anti-DNA but that of those studied only Paramecium and Pernnema nuclei were stained by anti-nucleohistone sera. The appearances are illustrated in figs. 12/1 - 12/20. The nuclei were never stained by normal sera. The differences between the staining reactions of anti-DNA and anti-nucleohistone sera on many species of protozoa cannot be explained by any gross difference in diffusibility of the antibodies nor by any differences in stability of the antigens as both types of antibody stained the nuclei of leucocytes in the blood films containing trypanosomes and the macrophages in the Toxoplasma gondii infected peritoneal exudate.

The tests to confirm the immunological specificity of staining by the antinuclear sera are shown in Table 12/2. These demonstrated that staining by "membranous" (anti-DNA) sera was prevented by predigestion with DNase or by absorption with DNA or nucleohistone and that the nuclear staining of Perenema by "homogeneous" (anti-nucleohistone) sera was prevented by predigestion with DNase and by absorption with nucleohistone only.

DISCUSSION

The nuclei of all species of Protozoa which were studied were stained with sera containing anti-DNA. This staining was immunologically specific since it was prevented by absorption of these antinuclear sera with purified DNA or by predigestion of the antigen

by DNase but not by other treatments (Table 12/2). This demonstrated that the antigenicity of the DNA in protozoal nuclei was identical with that in the nuclei of higher forms. Stollar et al. (1962c) had shown previously by a complement fixation technique that similar antigenic determinants for anti-DNA antinuclear sera were present in DNA extracted from normal and neoplastic mammalian tissue, many varieties of bacteria, several strains of bacteriophage, Euglena gracilis and Tobacco leaf, so that it was not surprising that the present investigation showed that protozoal DNA conformed to the general biological pattern.

Specific immunological staining of the nuclei of Paramecium and Peranema (Class Ciliophora) was produced by sera containing anti-nucleohistone (Table 12/2) but such antinuclear sera did not stain the other protozoa studied.

There have not been any previous investigations on the reaction of human antinuclear sera with protozoal nuclei. The present observations have shown that the DNA of the flagellates and Toxoplasma is in a different state of combination from the DNA of the ciliates and higher animals. The DNA of the more primitive protozoa may either be free or it may be combined with a protein other than histone. The first alternative appears more attractive since there is considerable evidence that bacterial and viral DNA is not associated with histone (Butler and Godson, 1963; Luzzati and Nicolaieff, 1963). The possibility that the DNA is combined with a non-histone protein

cannot, however, be excluded and further experiments are planned to resolve this problem by direct extraction and biochemical analysis since the only previous investigations on protozoal DNA have been concerned with analysis of its base composition (Jones and Thompson, 1963).

It is not possible at the present stage of this investigation to draw any firm conclusions on the relationship of antigenicity of nuclei to taxonomic classification of protozoa. Although there is a clear taxonomic difference between the organisms whose nuclei contain nucleohistone and those without this antigen (Table 12/1) the representatives of these groups which were tested also showed marked differences in biological behaviour, namely the antigen was present in the nuclei of the free living organisms but absent from the parasitic species. This unfortunate choice of species was dictated by the availability of organisms and further investigations are planned to cover many more species of protozoa and so to ascertain whether there is any relationship between nuclear antigens and taxonomy.

It is, however, remotely possible that absence of nucleohistone antigen might prove to be related to parasitic behaviour and it is interesting to speculate on how these characteristics might be related. Allfrey et al. (1963) have shown that DNA-primed enzymic RNA synthesis is more active in nuclei from which histone has been removed and they have suggested that histone may block the activity

of DNA or that it may have RNase activity. Either way, histone coupling would prevent "messenger" RNA synthesis on the histone-coupled DNA. If this hypothesis were translated into genetic terms, it would mean that histone coupling could block potential genetic expression on a gene and that redistribution of histone along the DNA chains could result in changes in activity of the genes at the corresponding loci. Thus, free living would only be possible if the organism had a wide range of genetic potentialities which could be adapted to the changing environment, whereas parasitism (or more rigid growth requirements) would imply less effective adaptation of the genetic potentialities. This lesser degree of adaptation in parasitic organisms could result from a smaller fund of genetic potentialities but it could equally well follow from a failure of modulation of genetic activity consequent upon rigid coupling of histone to particular parts of the DNA chain or from total absence of histone from the nucleus so that all the DNA was uncovered permanently. This last possibility could explain an association between uncoupled nuclear DNA and parasitism. It is fully realised that this speculation is not based on experimental evidence, but it is hoped that it might stimulate further investigations.

SUMMARY

Anti-DNA antinuclear sera stained the nuclei of all protozoa. Anti-nucleohistone antinuclear sera stained the nuclei of certain ciliates but failed to stain the nuclei of trypanosomes, Leishmania

donovani, Strogomonas or Toxoplasma. It is concluded that in the latter group of organisms the DNA is either free or combined with a non-histone protein. Possible explanations for the absence of the nucleohistone antigen from the latter group of protozoa (Taxonomic status or parasitic behaviour) are discussed.

SUMMARY

1. In the fluorescent antibody test for antinuclear antibodies variations were noted between the patterns of nuclear staining produced by sera from different "connective tissue disease" patients. The appearances could be explained by 4 fundamental patterns which might be present singly or in any combination. The patterns were named "homogeneous", "speckled", "nucleolar" and "membranous". They have been described in Chapter 1 and illustrated in figs. 1/1 - 1/4. Evidence was presented to demonstrate that the nuclear staining patterns were produced by sera containing different antibody systems.

2. "Homogeneous" nuclear staining was produced by sera containing an autoantibody to nucleohistone (DNA-histone).

3. "Speckled" nuclear staining was produced by sera containing an autoantibody to a saline soluble protein (sedimentation coefficient approximately 3.0S) normally present in mammalian cell nuclei.

4. The antigen responsible for "nucleolar" staining has not been identified but absorption experiments indicated that it was not RNA.

5. "Membranous" nuclear staining was produced by sera containing an autoantibody to DNA.

6. The serum factors were shown to be immunoglobulins (7.0S γ globulins or 19.0S macroglobulins) by ultracentrifugation and DEAE Sephadex chromatography. In view of the specificity of their reactions, they must be considered true antibodies.

7. The incidence of antinuclear antibodies in random hospital

patients (without evidence of autoimmune disease) detected by the fluorescent antibody test was shown to be 46.2% with undiluted serum, 26.6% at 1/4 serum dilution and 4% at 1/16 dilution. Nuclear staining was significantly commoner in middle aged women but it was less common and unrelated to age in men. It was not possible to determine whether weak staining was immunological, but these reactions were avoided in the subsequent clinical investigations by screening sera at 1/16 dilution.

8. The incidence and titres of various systems of antinuclear antibodies were investigated in the sera of 594 patients with "autoimmune diseases" or clinically related syndromes and the results have been summarised in Table 8/38. In general, the findings reflected the clinical severity of the disease as antinuclear antibodies were common (and often high titre) in systemic lupus erythematosus (82.5%) and progressive systemic sclerosis (78%) in the intermediate range in Sjögren's syndrome (62%) and discoid lupus erythematosus (35%) and relatively uncommon (and usually low titre) in rheumatoid arthritis (24%) and pernicious anaemia (18%). Antinuclear antibodies were not detected in small numbers of patients with polyarteritis nodosa and dermatomyositis despite widespread systemic involvement in these diseases; this suggests that the metabolic disturbance in these diseases may be different from that in the other "connective tissue diseases" and that they should not be classified within the same group.

The syndromes of the "connective tissue diseases" had been separated on the basis of differences in the clinical and morbid anatomical appearances; my observations on the antinuclear antibodies have shown analogous differences in the production of auto-antibodies as closely similar immunological responses were found in clinically related syndromes whereas marked differences were noted between the clinically less closely related syndromes.

My experience with the quantitative fluorescent antibody test for antinuclear antibodies has shown that it is convenient and gives reproducible results. I consider that it is the most suitable technique at present available for the detection of antinuclear antibodies. In clinical practice it has proved a valuable diagnostic aid.

9. Studies on 3 pregnancies in 2 women with systemic lupus erythematosus showed that the "homogeneous" antinuclear antibody had passed across the placenta and at birth the titres in the babies' sera were similar to those in the maternal sera. In each case, the antinuclear antibody was a 7.0S globulin. All 3 babies were healthy at birth and none developed any evidence of lupus erythematosus. Serial studies showed that the half life of the antinuclear antibodies were similar to those of antibodies to exogenous antigens and this suggested that the antinuclear antibodies were not reacting with their antigen <u>in vivo</u>. Antinuclear antibody could not be demonstrated in amniotic fluid, colostrum or milk.

10. Certain monospecific human antinuclear sera were used to trace the corresponding nuclear antigens in HeLa cells at various stages of mitosis. DNA, either free ("membranous" antigen) or combined with histone ("homogeneous" antigen), condensed in prophase and was incorporated completely into the chromosomes until it was diffused in the reforming nuclei of the daughter cells. The "speckled" antigen left the nucleus in early prophase and was dispersed within the cytoplasm before the onset of metaphase and ultimately returned to the nucleoli which were reforming in late telophase.

This demonstrated that at least two of the constituents of interphase nuclei were not present in the chromosomes and so were probably not important for inheritance. Furthermore, these results would explain the previous observations of loss of nuclear mass in prophase with recovery in telophase.

11. Human monospecific antinuclear sera have shown that the "speckled" and "nucleolar" antigens were absent from normal polymorphs but that all nuclear antigens were present in all other normal and leukaemic leucocytes. Leucocytes proved to be a relatively insensitive substrate for tests for human antinuclear antibodies.

High titre human antinucleolar sera proved specific stains for the detection of nucleoli in leucocyte nuclei, so that it was possible to follow the fate of these structures during maturation of leucocyte precursors.

12. The "membranous" antigen (DNA) was demonstrated in the nuclei

of all species which were examined but the "homogeneous" antigen (nucleohistone) was detected only in the more complex ciliates. The significance of this finding was discussed in relation to the taxonomy of Protozoa and to the capacity of organisms to adapt to changes in their environments.

13. The antinuclear antibodies have been shown to be valuable in the diagnosis of the "connective tissue diseases" as indicators of the underlying widespread loss of immunological tolerance. It is considered that these antibodies are of little importance in initiating tissue damage in these diseases but their role in potentiating the lesions has not yet been determined.

Human antinuclear antibodies have been introduced as specific histochemical reagents for 4 nuclear antigens. They have yielded cytochemical information on the structure of the nuclei of individual cells which could not have been obtained by any other technique at present available.

TABLE 2/1

THE DISTRIBUTION OF THE "HOMOGENEOUS" ANTIGEN IN VARIOUS TISSUES

All tissues were stained by the sandwich method using at least two "homogeneous" sera, one of which was always Dun. and the other usually Nel. In many cases, however, the preparations were stained by six different sera, all of which caused "homogeneous" nuclear staining on unfixed rat liver sections. In a few experiments the results were confirmed by staining with directly-conjugated Dun.

	Staining reaction with "homogeneous" antinuclear sera on nuclei	
Species	POSITIVE	NEGATIVE
Human	Peripheral blood leucocytes Peripheral blood chronic and subacute lymphatic and myeloid leukaemia leucocytes Thyroid acini and stroma (normal and thyrotoxic) (surgical specimen) Normal kidney (surgical specimen) Normal adrenal (surgical specimen) Normal skin (necropsy specimen) Normal liver (necropsy specimen) Desquamated buccal squames Carcinoma, female breast (surgical specimen) Fibroadenoma, female breast (surgical specimen)	Spermatozoa (ejaculated)
Rat	Liver Pancreas Brain Lymph node Kidney Leucocytes Sertoli cell and spermatogonia (impression smear) Nuclei isolated after homogenisation of rat liver in 0.22M sucrose	Spermatozoa (impression smear of testis)

TABLE 2/1 (Continued)

Species	POSITIVE	NEGATIVE
Guinea-pig	Liver Brain Lymph node Kidney Leucocytes	Spermatozoa (epididymal)
Mouse	Liver Leucocytes Follicular cells from ovarian follicles	Spermatozoa (epididymal)
Rabbit	Liver Kidney Leucocytes	
Ferret	Liver Leucocytes	
Hamster	Liver Leucocytes	
Cotton rat	Liver Leucocytes	
Hen	Liver Erythrocytes Leucocytes	

TABLE 2/1 (Continued)

Species	POSITIVE	NEGATIVE
Frog	Liver Erythrocytes Leucocytes	
Tissue culture	Hela cells Chicken fibroblasts Monkey kidney	Trachoma inclusion bodies in monkey kidney tissue culture
Protozoa (all as air dried unfixed smears)	Paramecium Peranema	Various species of trypanosomes Leishmania donovani Strigomonas Toxoplasma gondii

TABLE 2/2

EFFECT OF FIXATION ON THE "HOMOGENEOUS" ANTIGEN

(Substrate: cryostat sections of rat liver)

	FIXATIVE				
Conditions	Absolute acetone	Absolute ethanol	70% Ethanol	Absolute methanol	4% Formaldehyde
15°C. for 10 min.	++ typical "homogeneous" pattern	+ finely granular	± coarsely granular	...	negative
- 20°C. periods up to 1 hr	++ typical "homogeneous" pattern	++ typical "homogeneous" pattern	...	++ typical "homogeneous" pattern	...

Code: ++ Bright nuclear staining

+ Moderately bright nuclear staining

± Dull nuclear staining

Control sections (not subjected to fixation) ++

TABLE 2/3

EFFECT OF ENZYMIC DIGESTION ON THE "HOMOGENEOUS" ANTIGEN

(Substrate: acetone-fixed cryostat sections of rat liver)

	TREATMENT	
	Enzyme	Buffer control
DNase	Neg.	++
RNase	++	++
Trypsin	Neg. A	++
Chymotrypsin	Neg. A	++
Pepsin	Neg. B	± B

CODE

++ Bright nuclear staining

+ Moderately bright nuclear staining

± Dull nuclear staining

A Considerable autofluorescence but little upset of histological appearances.

B Gross autofluorescence and loss of much cytological detail.

TABLE 3/1

THE DISTRIBUTION OF THE "SPECKLED" ANTIGEN IN VARIOUS TISSUES

All tissues were stained by the sandwich method using at least two "speckled" sera, one of which was always Tra. and the other usually Rai. In many cases, however, the preparations were stained by six different sera, all of which caused pure "speckled" staining on unfixed rat liver sections. In a few experiments the results were confirmed by staining with directly-conjugated serum Tra.

Species	Staining reaction with "speckled" antinuclear sera on nuclei	
	POSITIVE	NEGATIVE
Human	Lymphocytes and Monocytes (normal blood film) All leukaemia leucocytes except some polymorph forms in chronic myeloid leukaemia Thyroid acini and stroma (normal and thyrotoxic) Normal kidney (surgical specimen) Normal adrenal (surgical specimen) Normal skin (necropsy specimen) Normal liver (necropsy specimen) Desquamated buccal squames Carcinoma, female breast (surgical specimen) Fibroadenoma, female breast (surgical specimen)	Polymorphonuclear leucocytes Some polymorph forms in chronic myeloid leukaemia Spermatozoa (ejaculated)
Rat	Liver Pancreas Brain Lymph node Kidney Leucocytes Sertoli cells and Spermatogonia (impression smear) Nuclei isolated after homogenisation of rat liver in 0.22M sucrose	Spermatozoa (impression smear)

TABLE 3/1 (Continued)

Species	POSITIVE	NEGATIVE
Guinea-pig	Liver Brain Lymph node Kidney Leucocytes	Spermatozoa (epididymal)
Mouse	Liver Follicular cells from ovarian follicles Leucocytes	Spermatozoa (epididymal)
Rabbit	Liver Kidney Leucocytes	
Ferret	Liver Leucocytes	
Hamster	Liver Leucocytes	
Cotton rat	Liver Leucocytes	
Hen		Liver Erythrocytes Leucocytes

TABLE 3/1 (Continued)

Species	POSITIVE	NEGATIVE
Frog		Liver Erythrocytes Leucocytes
Tissue culture	Hela cells Chicken fibroblasts Monkey kidney	Trachoma inclusion bodies in monkey kidney tissue culture
Protozoa (unfixed air-dried smears)		Various species of trypanosomes, Leishmania donovani, Strigomonas, Toxoplasma gondii, Paramecium Peranema

TABLE 3/2

EFFECT OF FIXATION ON THE "SPECKLED" ANTIGEN

(Substrate: cryostat sections of rat liver)

	FIXATIVE				
Conditions	Absolute acetone	Absolute ethanol	70% ethanol	Absolute methanol	4% Formaldehyde
15°C. for 10 min.	Some clumping but still obviously ++ "speckled"	Coarse ± clumping	Negative	.....	Negative
- 20°C. for 1 hr	very little clumping. obviously ++ "speckled"	Coarse + clumping	....	very little ++ clumping still obviously "speckled"	....

Code: ++ Bright nuclear staining

\+ Moderately bright nuclear staining

± Dull nuclear staining

Control sections (not subjected to fixation) ++

TABLE 3/3

EFFECT OF ENZYMIC DIGESTION ON THE "SPECKLED" ANTIGEN

(Substrate: acetone-fixed cryostat sections of rat liver)

	TREATMENT	
	Enzyme	Buffer control
DNase	++	++
RNase	++	++
Trypsin	Neg. A	++
Chymotrypsin	Neg. A	++
Pepsin	Neg. B	± B

CODE

++ Bright nuclear staining

\+ Moderately bright nuclear staining

± Dull nuclear staining

A Considerable autofluorescence but little upset of histological appearances

B Gross autofluorescence and loss of much cytological detail

TABLE 3/4

STAINING OF ISOLATED RAT LIVER NUCLEI

Staining serum	Unextracted nuclei	Nuclei extracted with 0.15M - NaCl (pH 7.4) 5 min. - 3 hr
"Speckled"	+++ (Fig. 3/1)	Negative
"Homogeneous"	+++ (Fig. 3/2)	+++
Normal	Negative (Fig. 3/3)	Negative

CODE: +++ Bright nuclear staining

TABLE 3/5

ULTRA-CENTRIFUGATION OF "SPECKLED" ANTIGEN USING RADIOACTIVE MARKERS

Fractions were numbered consecutively from the bottom of the tube upwards

Fraction No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Reciprocal of titre of "speckled" antigen	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	? 1	Neg.	16	16	16	Neg.	Neg.
^{125}I Ovalbumin (3.5S) (counts/min.)	278	391	546	773	1,040	1,577	2,309	2,688	2,593	2,242	1,914	1,938	1,797	1,858
^{131}I Mercaptoalbumin (counts/min.)	305	673	1,055	1,984	3,446	4,821	4,089	2,444	1,073	537	362	265	252	256

NOTE: The failure of the ^{125}I counts to drop more in fractions 9 - 14 is due to free iodine which tends to split off ovalbumin for some days after iodination.

TABLE 4/1

THE DISTRIBUTION OF THE "NUCLEOLAR" ANTIGEN IN VARIOUS TISSUES

All tissues were stained by the sandwich method using unabsorbed antinucleolar sera (Mui. or Scr.) as it was possible to identify nucleoli from their morphology in the presence of dull staining of the rest of the nucleus. In a few cases the results were confirmed with MUI. and SCR. or by directly-conjugated MUI.

Species	Staining reaction with antinucleolar sera	
	POSITIVE	NEGATIVE
Human	Lymphocytes and Monocytes (normal blood film) All leukaemic leucocytes except most stab and polymorph forms in chronic myeloid leukaemia Thyroid (normal and thyrotoxic) (surgical specimen) Normal kidney (surgical specimen) Normal adrenal (surgical specimen) Normal skin (necropsy specimen) Normal liver (necropsy specimen) Desquamated buccal squames Carcinoma, female breast (surgical specimen) Fibroadenoma, female breast (surgical specimen)	Polymorphonuclear leucocytes Stab and polymorph forms in chronic myeloid leukaemia Spermatozoa (ejaculated)
Rat	Liver Pancreas Brain Lymph node Kidney Leucocytes Sertoli cells and spermatogonia (impression smear) Nuclei isolated after homogenisation of rat liver in 0.22M sucrose	Spermatozoa (impression smear)

TABLE 4/1 (Continued)

Species	POSITIVE	NEGATIVE
Guinea-pig	Liver Brain Lymph node Kidney Leucocytes	Spermatozoa (epididymal)
Mouse	Liver Follicular cells from ovarian follicles Leucocytes	Spermatozoa (epididymal)
Rabbit	Liver Kidney Leucocytes	
Ferret	Liver Leucocytes	
Hamster	Liver Leucocytes	
Cotton rat	Liver Leucocytes	
Hen	Liver	Erythrocytes
Frog	Liver	Erythrocytes

TABLE 4/1 (Continued)

Species	POSITIVE	NEGATIVE
Tissue cultures	Hela cells Chicken fibroblasts	
	Monkey kidney	Trachoma inclusion bodies in monkey kidney tissue cultures
Protozoa		Various species of Trypanosomes Leishmania donovani Strigomonas Toxoplasma gondii Paramecium Peranema

TABLE 4/2

EFFECT OF FIXATION ON THE "NUCLEOLAR" ANTIGEN

(Substrate: cryostat sections of rat liver; Staining system: MUI.)

Conditions	FIXATIVE				
	Absolute acetone	Absolute ethanol	70% ethanol	Absolute methanol	4% Formaldehyde
15°C for 10 min.	++	+ -	Negative	....	Negative
- 20°C for 1 hr	++	+	....	++	....

Code: +++ Intense nucleolar staining

++ Bright nucleolar staining

+ Moderately bright nucleolar staining

± Dull nucleolar staining

Control sections (not subjected to fixation) ++

TABLE 4/3

EFFECTS OF ENZYMIC DIGESTION ON THE "NUCLEOLAR" ANTIGEN

	TREATMENT	
	Enzyme	Buffer control
DNase	++	++
RNase	neg.	++
Trypsin	neg. A	++
Chymotrypsin	neg. A	++
Pepsin	neg. B	±B

CODE

++ Bright nucleolar staining

\+ Moderately bright nucleolar

± Dull nucleolar staining

A Considerable autofluorescence but little upset of histological appearances

B Gross autofluorescence and loss of much cytological detail

TABLE 4/4

STAINING OF ISOLATED RAT LIVER NUCLEI BY ANTINUCLEOLAR ANTIBODY

Staining serum	Unextracted nuclei		Nuclei extracted with 0.15M - NaCl (pH 7.4) for 30 min.	
	Nucleus	Nucleolus	Nucleus	Nucleolus
Mui.	+	+++	+	negative
Wax. (Homogeneous)	+++	negative	+++	negative
Bec. (Normal)	negative	negative	negative	negative

Code: +++ Bright staining

+ Dull staining

TABLE 5/1

DISTRIBUTION OF THE "MEMBRANOUS" ANTIGEN IN VARIOUS TISSUES

All tissues were stained by the sandwich method using two "membranous" antinuclear sera, one of which was always McDu. Directly-conjugated staining was not attempted because the "membranous" antinuclear sera were in short supply.

	Staining reaction with "membranous" antinuclear sera on nuclei	
Species	POSITIVE	NEGATIVE
Human	Lymphocytes, monocytes and polymorphs (normal blood) All leukaemic leucocytes Thyroid (normal and thyrotoxic) (surgical specimen) Normal kidney (surgical specimen) Desquamated buccal squames Carcinoma, female breast (surgical specimen)	Spermatozoa (ejaculated)
Rat	Liver Pancreas Brain Lymph node Kidney Leucocytes Sertoli cells and spermatogonia (impression smear)	Spermatozoa (impression smear and epididymal)
Hen	Liver Leucocytes Erythrocytes	

TABLE 5/1 (Continued)

Species	POSITIVE	NEGATIVE
Frog	Liver Leucocytes Erythrocytes	
Protozoa	Various species of trypanosomes Toxoplasma gondii Strigomonas Paramecium Peranema	

TABLE 5/2

EFFECT OF FIXATION ON THE "MEMBRANOUS" ANTIGEN

(Substrate: cryostat sections of rat liver)

	FIXATIVE					
Conditions	Absolute acetone	Absolute ethanol	70% ethanol	Absolute methanol	4% Formaldehyde	PALADE'S Fixative (osmic acid)
15°C for periods up to 1 hr	+++	+++	+++	....	+++	....
- 20°C for periods up to 1 hr	+++	+++	....	+++	....	....
Paraffin embedded tissues	....	....	....	....	+++	+++

Code: +++ Bright nuclear staining

Control sections (not subjected to fixation) +++

TABLE 5/3

EFFECT OF ENZYMIC DIGESTION ON THE "MEMBRANOUS" ANTIGEN

(Substrate: acetone-fixed cryostat sections of rat liver)

	TREATMENT	
	Enzyme	Buffer control
DNase	negative	+++
RNase	+++	+++
Trypsin	+++ A	+++
Chymotrypsin	+++ A	+++
Pepsin	+++ B	+++ B

CODE

+++ Bright nuclear staining

A Considerable autofluorescence but little upset of histological appearances

B Gross autofluorescence and loss of much cytological detail.

TABLE 5/4

PREVIOUS REPORTS OF ANTI-DNA AUTOANTIBODIES

IN "CONNECTIVE DISEASE" SERA

Complement Fixation

Coppellini et al.	(1957)
Robbins et al.	(1957)
Seligmann	(1957)
Seligmann and Milgrom	(1957)
Pearson et al.	(1958)
Stollar and Levine	(1961, 1962)
Stollar et al.	(1962a, b and c)
Williams and Bollum	(1963)

Precipitin Tests

Coppellini et al.	(1957)
Seligmann	(1957)
Deicher et al.	(1959)
Lima et al.	(1961)
Anderson et al.	(1962)

Haemagglutination

Miescher and Strässle (1957)

Bentonite flocculation

Kayhoe et al. (1960)

Passive cutaneous anaphylaxis

Deicher et al. (1960)

Cutaneous auto-sensitivity

Levin and Pinkus (1961)

Inhibitation of DNA polymerase

Williams and Bollum (1963)

TABLE 6/1

ULTRACENTRIFUGATION OF ANTINUCLEAR SERA

Each serum was mixed with a small quantity of ^{131}I normal human 7.0S globulin before layering on a sucrose density gradient

	FRACTION												
	1	2	3	4	5	6	7	8	9	10	11	12	13
Abr. counts/min. nuclear fluorescence	590 neg.	3,583 1/1 S	9,990 1/4 S	14,721 1/4 S	4,670 1/1 S	160 neg.							
Tra. counts/min. nuclear fluorescence	- neg.	- neg.	- neg.	- neg.	72 neg.	920 1/1 S	4,664 1/4 S	2,576 1/16 S	434 1/1 S				
Dun. counts/min. nuclear fluorescence	- neg.	- neg.	- neg.	- neg.	80 neg.	546 neg.	4,453 1/16 H	9,120 1/4 H	5,015 1/16 H	916 1/1 H			
Mai. counts/min. nuclear fluorescence nucleolar fluorescence	- neg. neg.	- neg. neg.	- neg. neg.	127 neg. neg.	1,346 neg. 1/4 N	5,048 ± H 1/16 N	7,024 neg. 1/16 N	2,315 neg. 1/4 N	297 neg. neg.				
Dev. counts/min. nuclear fluorescence	- neg.	- neg.	- neg.	17 neg.	78 neg.	454 +S	3,324 +±S	6,526 ++S	3,834 ++S	591 ?			
Don. counts/min. nuclear fluorescence	- neg.	- neg.	- neg.	14 neg.	61 neg.	341 neg.	2,114 ?	6,396 +S	4,463 ±S	692 neg.			
McCor. counts/min. nuclear fluorescence	- neg.	- neg.	- neg.	14 neg.	61 neg.	345 neg.	2,568 neg.	7,398 ±H	5,489 neg.	751 neg.			
McDon. counts/min. nuclear fluorescence	- neg.	- neg.	- neg.	- neg.	- neg.	145 neg.	712 neg.	2,466 +S	6,715 ++S	8,926 ++S	5,268 +S	1,458 neg.	670 neg.
McLar. counts/min. nuclear fluorescence	- neg.	- neg.	- neg.	- neg.	13 neg.	412 ?	1,228 ++H	4,903 ++H	4,579 ±H	643 neg.			

H = "Homogeneous" nuclear staining; S = "Speckled" nuclear staining; N = "Nucleolar nuclear staining

TABLE 6/2

ULTRACENTRIFUGATION OF ANTINUCLEAR SERUM McDAI.

Normal human 7.0S globulin labelled with ^{131}I was used as marker

	FRACTION									
	1	2	3	4	5	6	7	8	9	10
McDai. counts/min.	-	-	13	59	201	1,016	3,514	9,546	3,310	529
Nuclear fluorescence	neg.	neg.	neg.	+ H	+ H	± H	neg.	neg.	neg.	neg.

H = "Homogeneous" nuclear staining

TABLE 6/3

DEAE SEPHADEX CHROMATOGRAPHY OF ANTINUCLEAR SERA

Serum	Fraction A (γ globulin)	Fraction B (β globulin)	Fraction C (Albumin 1 and 2 globulin	Fraction D (2 and β globulin)
Tra.	"Speckled"	nil	nil	nil
Dun.	"Homogeneous"	nil	nil	nil
Mar.	"Homogeneous"	nil	nil	nil
Mui.	"Nucleolar" "Homogeneous"	nil	nil	nil

TABLE 7/1

AGE INCIDENCE OF TITRES OF NUCLEAR STAINING IN 500 BLOOD GROUPING SERA

		TITRE						
Age Group (yrs)	Sex	1/1	+ 1/4 –	1/4	+ 1/16 –	1/16	1/64	Total
0 +	M	–	– –	– –	– –	– –	– –	–
	F	1	– –	– –	– –	– –	– –	1
10 +	M	–	1H –	– –	– –	– –	– –	1
	F	1	1H 1S	1H –	– –	– –	– –	4
20 +	M	4	1H –	– –	– –	– –	– –	5
	F	4	2H –	1H –	– –	2H –	– –	9
30 +	M	3	5H –	2H –	– –	– –	– –	10
	F	11	7H 2S	5H –	– –	1H –	– –	26
40 +	M	7	2H 2S	– –	– –	– 1S	– –	12
	F	21	6H 1S	3H –	– –	2H –	– –	33
50 +	M	8	1H 4S	2H 2S	– –	1H –	– –	18
	F	12	4H 2S	2H 2S	– –	– –	– –	22
60 +	M	7	– 4S	5H –	2H –	– –	– –	19
	F	11	7H 3S	8H 2S	1H 1S	3H –	– –	36
70 +	M	–	3H 1S	– –	– –	– –	– –	4
	F	5	4H 2S	4H –	– –	1H 3S	1H –	20
80 +	M	–	1H –	1H –	– –	– –	– –	2
	F	3	2H –	2H 2S	– –	– –	– –	9
Total		98	47H 22S	36H 8S	4H 1S	10H 4S	1H –	
Incidence in whole series		19.6%	13.6%	8.8%	1.0%	2.8%	0.2%	

H = "Homogeneous" staining

S = "Speckled" staining

TABLE 7/2

AGE INCIDENCE OF NUCLEAR STAINING IN FEMALES

Comparison of tests at various levels of sensitivity

Age (yrs)	No.	All titres	+ 1/4 or greater -	1/4 or greater	+ 1/16 or greater -	1/16 or greater
0 +	1	1 (100%)	0 (-)	0 (-)	0 (-)	0 (-)
10 +	4	4 (100%)	3 (75%)	1 (25%)	0 (-)	0 (-)
20 +	18	9 (50%)	5 (27.7%)	3 (16.7%)	2 (11.1%)	2 (11.1%)
30 +	47	26 (55.3%)	15 (31.9%)	6 (12.8%)	1 (2.1%)	1 (2.1%)
40 +	75	33 (44.0%)	12 (16.0%)	5 (6.7%)	2 (2.7%)	2 (2.7%)
50 +	51	22 (43.1%)	10 (19.6%)	4 (7.8%)	0 (-)	0 (-)
60 +	54	36 (66.7%)	25 (46.3%)	15 (27.8%)	5 (9.3%)	3 (5.5%)
70 +	21	20 (95.2%)	15 (71.4%)	9 (42.9%)	5 (23.8%)	5 (23.8%)
80 +	9	9 (100%)	6 (66.7%)	4 (44.4%)	0 (-)	0 (-)
Total	280	160 (57%)	91 (32%)	47 (17%)	15 (5.3%)	13 (4.6%)
χ^2		14.926	27.761	24.29%	21.406	23.496
P		0.1 - 0.05	<0.01	<0.01	<0.01	<0.01

TABLE 7/3

AGE INCIDENCE OF NUCLEAR STAINING IN MALES

Comparison of tests at various levels of sensitivity

Age (yrs)	No.	All titres	+ 1/4 or greater -	1/4 or greater	+ 1/16 or greater -	1/16 or greater
0 +	0	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)
10 +	8	1 (12.5%)	1 (12.5%)	0 (-)	0 (-)	0 (-)
20 +	25	5 (20.0%)	1 (4.0%)	0 (-)	0 (-)	0 (-)
30 +	19	10 (52.6%)	7 (36.8%)	2 (10.5%)	0 (-)	0 (-)
40 +	28	12 (42.9%)	5 (17.9%)	1 (3.6%)	1 (3.6%)	1 (3.6%)
50 +	54	18 (33.3%)	10 (18.5%)	5 (9.3%)	1 (1.9%)	1 (1.9%)
60 +	49	19 (38.8%)	12 (24.5%)	8 (16.3%)	3 (6.1%)	0 (-)
70 +	28	4 (14.3%)	4 (14.3%)	0 (-)	0 (-)	0 (-)
80 +	9	2 (22.2%)	2 (22.2%)	1 (11.1%)	0 (-)	0 (-)
Total	220	71 (32.0%)	40 (19.0%)	17 (8.0%)	5 (2.2%)	2 (0.9%)
χ^2		9.662	7.691	10.647	5.516	3.993
P		0.30	0.50	0.30	0.70	0.90 - 0.80

TABLE 8/1

LABORATORY INVESTIGATIONS ON PATIENTS WITH VARIOUS DISEASES

	Fluorescent antibody test for antinuclear antibodies	L.E. cell test	Rheumatoid factor test	Anti-thyroid antibody test	Anti-gastric antibody test	Autoimmune complement fixation reaction	Precipitin test for antitissue antibodies
Systemic lupus erythematosus	+	+	....	....	+	....	+
Discoid lupus erythematosus	+	+	+	....	....	....	+
Progressive systemic sclerosis	+	+	....	....	....	....	+
Sjögren's syndrome	+	+	+	+	+	+	+
Rheumatoid arthritis	+	....	....	....	....	....	+
Pernicious anaemia	+	....	+	+	+	....	+

TABLE 8/2

INCIDENCE OF DIFFERENT TYPES OF ANTINUCLEAR ANTIBODIES IN SIE PATIENTS FROM GLASGOW AND LEEDS

		Antinuclear antibody test					
Source	No.	No. Negative	No. Positive	"Homogeneous"	"Membranous"	"Speckled"	"Nucleolar"
Glasgow	46	10	36	24	3	9	1
Leeds	34	4	30	19	-	11	1
Total	80	14	66	43	3	20	2

TABLE 8/3

INCIDENCES OF DIFFERENT TYPES OF PRECIPITATING AUTOANTIBODIES IN SIE PATIENTS FROM GLASGOW AND LEEDS

		Precipitating autoantibodies							
Source	No.	No. Negative	No. Positive	Anti-SjD	Anti-SjT	Anti-Lup	Anti-DNA	Anti-RA	Not identified
Glasgow	46	21	25	6	1	5	3	1	11
Leeds	34	14	20	9	3	7	-	-	5
Total	80	35	45	15	4	12	3	1	16

The results in Table 8/3 are reproduced with permission from Dr J. R. Anderson who made the experimental observations.

TABLE 8/4

RELATIONSHIP BETWEEN ANTINUCLEAR ANTIBODIES AND PRECIPITATING AUTOANTIBODIES IN SLE

Antinuclear antibodies (all types)	Precipitating autoantibodies (all types)	
	Negative	Positive
Negative	7	7
Positive	28	38

TABLE 8/5

THE RELATIONSHIP OF SYSTEMS OF ANTINUCLEAR ANTIBODIES

TO SYSTEMS OF PRECIPITATING AUTOANTIBODIES IN SLE

		Precipitating autoantibodies							
Antinuclear Antibody	No.	Negative (35)*	Positive (45)*	Anti-SjD (15)	Anti-SjT (4)	Anti-Lup (12)	Anti-DNA (3)	Anti-RA (1)	Not identified (16)
"Homogeneous"	43	23	20	9	1	5	-	1	7
"Membranous"	3	-	3	-	-	-	3	-	-
"Speckled"	20	5	15	6	3	7	-	-	3
"Nucleolar"	2	1	1	1	1	-	-	-	-
Negative	14	7	7	1	-	-	-	-	6

* antinuclear antibody associated with "homogeneous" antinuclear antibody in both cases

TABLE 8/6

ANTIGASTRIC ANTIBODIES IN SYSTEMIC LUPUS ERYTHEMATOSUS

Source	Antigastric Antibodies	Antinuclear Antibodies	
		PRESENT	ABSENT
Glasgow	PRESENT (5)	4	1
	ABSENT (35)	29	6
Leeds	PRESENT (1)	1	-
	ABSENT (32)	28	4

TABLE 8/7

PUBLISHED REPORTS OF THE INCIDENCE OF ANTINUCLEAR ANTIBODIES IN SYSTEMIC LUPUS ERYTHEMATOSUS

Fluorescent antibody technique

Bardawil et al. (1958)	5/5
Calabresi et al. (1959)	15/15
Alexander et al. (1960)	12/12
Baugh et al. (1960)	35/39
Hall et al. (1960)	22/22
Hijmans et al. (1961)	64/65
Kratchko (1961)	31/34
Mandema et al. (1961)	51/56
Pollak et al. (1961)	45/51
Rothfield et al. (1961)	43/53
Weir et al. (1961)	62/63
Widelock et al. (1961)	43/51
Barnes (1962)	21/25
Fennell et al. (1962)	5/5
Thompson (1962)	28/33
Thivolet et al. (1963)	29/33

Other immunological techniques

(a) Anti-nucleohistone:

Holman and Kunkel (1957)	precipitin	
Miescher and Strässle (1957)	T.R.C.	3/5
Christian et al. (1958)	Latex	19/24
Friou (1958)	Fluorescent spot	35/35
Fessell et al. (1959)	Latex	25/37

TABLE 8/7 (Continued)

Goodman and Bowser (1959)	T.R.C.	19/22
Holman and Deicher (1959)	precipitin	
Scalleter et al. (1960)	C.F.T.	9/10
Dubois et al. (1961)	Latex	24/154
Edington and Walford (1961)	C.F.T.	7/14
Lima et al. (1961)	T.R.C.	18/21
Rothfield et al. (1961)	C.F.T.	23/35
Townes et al. (1963a)	C.F.T.	36/39
(b) Anti-DNA:		
Ceppellini et al. (1957)	C.F.T. precipitin	1/8
Robbins et al. (1957)	C.F.T.	4/5
Miescher and Strassle (1957)	T.R.C.	3/5
Pearson et al. (1958)	C.F.T.	7/20
Deicher et al. (1959)	precipitin	8/14
Jokinen and Makitalo (1959)	T.R.C.	8/9
Bozievich et al. (1960)	Bentonite flocculation	8/8
Deicher et al. (1960)	P.C.A.	9/12
Kayhoe et al. (1960)	Bentonite flocculation	13/13
Lima et al. (1961)	precipitin	9/21
	T.R.C.	17/21
Seligmann (1962)	precipitin untreated	28/32
	remission	26/79
Williams and Bollum (1963)	inhibition of enzymic DNA synthesis	12/14
Stollar et al. (1962c)	C.F.T.	11/37

TABLE 8/7 (Continued)

(c) Anti-histone:		
Holman et al. (1959)	C.F.T.	....
Rothfield et al. (1961)	C.F.T.	5/35
(d) Anti-soluble protein:		
Holman et al. (1959)	C.F.T.	....

TABLE 8/8

THE INCIDENCE OF ANTINUCLEAR ANTIBODIES IN PATIENTS WITH DISCOID LUPUS ERYTHEMATOSUS

	Antinuclear antibodies						
	Number tested	Number positive	Incidence %	"Homogeneous"	"Speckled"	"Nucleolar"	H:S ratio
Total series	137	48	35	33	15	1	2.2
Leeds Group	120	42	35	29	13	-	2.2
Females	83	32	38.5	22	10	-	2.2
Males	37	10	27	7	3	-	2.3
Glasgow Group	17	6	35	4	2	1	2.0

TABLE 5/9

CLINICAL AND LABORATORY ABNORMALITIES IN

120 DISCOID LUPUS ERYTHEMATOSUS PATIENTS FROM LEEDS

ABNORMALITIES		Males (37)	Females (83)	Total (120)
Chilblains		4	24	28
Raynaud's phenomenon		1	16	17
Joint pains		5	27	32
E.S.R. (Westergren) Males> 10; Females> 20 mm./hr		4	20	24
Haemoglobin <11.0g./100 ml.		1	21	22
W.B.C. <5,000/cu.mm.		2	13	15
Platelet count <150,000/cu.mm.		2	4	6
Total serum globulin> 3.0g./100 ml.		10	24	34
Serum electrophoresis				
	↑ γ	3	9	12
	↑ α_2	-	3	3
	↑ β	-	1	1
Direct Coombs Test		1	2	3
L. E. cell test		-	2	2
Rheumatoid factor test		6	13	19
Precipitin reaction	anti-SjT	-	4	4
	unidentified	-	1	1
Wassermann reaction		1	5	6
Cryoglobulins		0/2	1/21	1/23
Cold agglutinins		0/5	2/19	2/24

TABLE 8/10

INCIDENCE OF ANTINUCLEAR ANTIBODIES IN DISCOID LUPUS ERYTHEMATOSUS

PATIENTS WITH OTHER ABNORMALITIES

ABNORMALITY	Test for antinuclear antibodies						Comparison with remainder of series	
	Neg.	1/16	1/64	1/256	1/1000	No. Pos.	χ^2	P
Chilblains	9	6H 5S	5H -	1H 1S	- 1S	19	15.5	0.001
Raynaud's phenomenon	6	2H 4S	3H -	1H -	- 1S	11	6.013	0.02
Joint pains	15	8H 2S	5H -	1H 1S	- -	17	9.802	0.01
E.S.R. Males >10 mm./hr Females >20	9	5H 4S	3H -	1H 2S	- -	15	8.519	0.01
Haemoglobin <11.0g./100 ml.	14	1H 2S	4H -	- 1S	- -	8	0.01	0.95
W.B.C. <5,000/cu.mm.	5	2H 1S	6H -	- 1S	- -	10	6.05	0.02
Platelet count <150,000/cu.mm.	2	- 2S	1H -	- 1S	- -	4	....	....

TABLE 8/10 (Continued)

ABNORMALITY	Test for antinuclear antibodies						Comparison with remainder of series	
	Neg.	1/16	1/64	1/256	1/1000	No. Pos.	χ^2	P
Total serum globulin > 3.0g./100 ml.	14	7H 5S	4H -	1H 2S	- 1S	20	10.42	0.001
Serum electrophoresis ↑ γ	6	- 2S	1H -	1H 1S	- 1S	6	6.879	0.01
↑ α_2	1	1H -	1H -	- -	- -	2	....	....
↑ β	-	- -	1H -	- -	- -	1	....	....
Direct Coombs Test	-	- 1S	1H -	1H -	- -	3	....	....
L. E. cell test	-	- -	2H -	- -	- -	2	....	....

TABLE 8/10 (Continued)

ABNORMALITY	Test for antinuclear antibodies						Comparison with remainder of series	
	Neg.	1/16	1/64	1/256	1/1000	No. Pos.	χ^2	P
Rheumatoid factor test	7	3H 3S	3H -	1H 2S	- -	12	6.47	0.01
Precipitin anti-SjT	-	- 1S	- -	- 2S	- 1S	4	....	....
Precipitin unidentified	1	- -	- -	- -	- -	-	....	....
Wasserman reaction	1	- 1S	3H -	1H -	- -	5	....	....

TABLE 8/11

RELATIONSHIP BETWEEN ANTINUCLEAR ANTIBODIES AND CHILBLAINS

IN LEEDS DISCOID LUPUS ERYTHEMATOSUS PATIENTS

	Number of patients	Antinuclear antibody test		
		No. Positive	"Homogeneous"	"Speckled"
Chilblains	28	19	12	7
No Chilblains	92	23	17	6
TOTAL	120	42	29	13

The difference between the H:S ratios for these groups is not significant. $\chi^2 = 0.17$: P = 0.7

TABLE 8/12

RELATIONSHIP BETWEEN ANTINUCLEAR ANTIBODIES AND RAYNAUD'S PHENOMENON IN LEEDS DISCOID LUPUS ERYTHEMATOSUS PATIENTS

	Number of patients	Antinuclear antibody test		
		No. Positive	"Homogeneous"	"Speckled"
Raynaud's phenomenon	17	11	6	5
No Raynaud's phenomenon	103	31	23	8
TOTAL	120	42	29	13

The difference between the H:S ratios for these 2 groups is not significant ($\chi^2 = 0.69$: $0.5 > P > 0.3$)

TABLE 8/13

RELATIONSHIP BETWEEN ANTINUCLEAR ANTIBODIES AND JOINT PAINS IN LEEDS DISCOID LUPUS ERYTHEMATOSUS PATIENTS

	Number of patients	Antinuclear antibody test		
		No. Positive	"Homogeneous"	"Speckled"
Joint pains	32	17	14	3
No Joint pains	88	25	15	10
TOTAL	120	42	29	13

The difference between the H:S ratios for these 2 groups is not significant ($\chi^2 = 1.35$: $P = 0.2$)

TABLE 8/14

RELATIONSHIP BETWEEN ANTINUCLEAR ANTIBODIES AND RHEUMATOID FACTOR IN LEEDS DISCOID LUPUS ERYTHEMATOSUS PATIENTS

	Number of patients	Antinuclear antibody test		
		No. Positive	"Homogeneous"	"Speckled"
Rose Waaler titre $\geq$ 1:16	19	12	7	5
Rose Waaler titre $<$ 1:16	101	30	22	8
TOTAL	120	42	29	13

The difference between the H:S ratios for the 2 groups is not significant ($\chi^2 = 0.337$: $0.7 > P > 0.5$)

TABLE 8/15

RELATIONSHIP BETWEEN ANTINUCLEAR ANTIBODY AND ROSE-WAALER TITRE IN LEEDS DISCOID LUPUS ERYTHEMATOSUS PATIENTS

Rose-Waaler Titre		Antinuclear antibody titre				
		Neg.	1/16	1/64	1/256	1/1000
	1/1000	1	- -	- -	- 1S	- -
	1/512	1	- -	1H -	- 1S	- -
	1/256	-	- 1S	- -	- -	- -
	1/128	-	- -	1H -	- -	- -
	1/64	1	1S -	- -	1H -	- -
	1/32	1	- 1S	- -	1H -	- -
	1/16	3	3H -	1H -	- -	- -
	Neg.	71	12H 6S	9H 1S	1H -	- 1S

TABLE 8/16

SUMMARY OF CLINICAL FEATURES OF 4 WOMEN WITH DISCOID LUPUS ERYTHEMATOSUS AND ERYTHEMA MULTIFORME-LIKE LESIONS

All patients showed erythrocyanosis and chilblains

Case	1	2	3	4
Age (years)	48	72	32	31
Raynaud's phenomenon	-	-	+	-
Discoid lupus erythematosus	legs, face and hands	face and hands	face	face
Duration of lupus erythematosus (years)	13 years	16 years	4 years	5 years
Duration of erythema multiforme-like lesions (years)	20 years	11 years	1 year	4 years
Site of erythema multiforme-like lesions	legs and chest	arms, legs, face and neck	arms and legs	arms and legs

TABLE 8/17

RESULTS OF INVESTIGATIONS ON 4 PATIENTS WITH DISCOID LUPUS ERYTHEMATOSUS AND ERYTHEMA MULTIFORME-LIKE LESIONS

Case	1	2	3	4
E.S.R. (Westergren)	49	15	10	26
Plasma proteins total)	8.2	7.2	7.4	6.5
albumin) g./100 ml.	4.0	4.3	3.9	3.2
globulin)	4.2	2.9	3.5	3.3
Serum-γ-globulin	raised	normal	raised	normal
Haemoglobin (100% = 13.8 g./100 ml.)	66%	70%*	88%	
White cell count (/cu.mm.)	3,300	2,200	15,100	
Platelets (/cu.mm.)	120,000	151,000	209,000	
Wasserman reaction	-	-	-	-
Coombs test	-	-	-	-
Rheumatoid factor titre (reciprocal)	1024	32	1024	512
Cryoglobulins	-	-	-	-
Cold agglutinins	-	-	-	-
L. E. cells (Zimmer and Hargraves, 1952)	-	-	-	-

TABLE 8/17 (Continued)

Case	1	2	3	4
Antinuclear factor (Beck, 1961b)	speckled	speckled	speckled	speckled
" " titre (reciprocal)	256	16	1000	256
Precipitin to tissue extracts (Anderson et al., 1961b)	anti-SjT	anti-SjT	anti-SjT	anti-SjT
" titre (reciprocal)	256	256	64	64
Fluorescent test for antibodies to gastric parietal cells	neg.	neg.	neg.	neg.
Urine (side room tests)	normal	normal	normal	normal
Radiography of chest	normal	normal	normal	normal
E. C. G.	normal	normal	normal	normal

* Further investigations revealed that the patient had pernicious anaemia

Titres are shown as reciprocals in this table.

TABLE 8/18

ANTINUCLEAR ANTIBODIES IN PROGRESSIVE SYSTEMIC SCLEROSIS

Comparison of incidence in Leeds and Glasgow patients

	Number of patients	Number with all types of antinuclear antibody	"Homogeneous"	"Speckled"	"Nucleolar"
Leeds	22	17	12	5	3
Glasgow	10	8	5	3	3
TOTAL	32	25 (78%)	17	8	6

TABLE 8/19

PRECIPITATING AUTOANTIBODIES IN PROGRESSIVE SYSTEMIC SCLEROSIS

Comparison of incidence in Leeds and Glasgow patients

	No. of patients	Sera containing precipitins	Anti-SjT	Anti-SjD	Anti-Lap	Anti-DNA	Not identified
Leeds	22	1	-	-	-	-	1
Glasgow	10	5	-	4	2	-	2
TOTAL	32	6	-	4	2	-	3

The results in table 8/19 are reproduced with permission from Dr J. R. Anderson who made the experimental observations.

TABLE 9/20

RELATIONSHIP BETWEEN SUBSETS OF ANTINUCLEAR AND PRECIPITATING AUTOANTIBODIES IN PROGRESSIVE SYSTEMIC SCLEROSIS

		Precipitating autoantibodies							
Antinuclear Antibody	No.	No. Negative	No. Positive	Anti-SjD (4)	Anti-SjT (-)	Anti-Jo (2)	Anti-DNA (-)	Anti-RA (-)	Not identified (3)
"Homogeneous"	17	15	2	2	-	1	-	-	1
"Speckled"	8	4	4	2	-	1	-	-	2
"Nucleolar"	6	4	2	2	-	1	-	-	1
"Membranous"	-	-	-	-	-	-	-	-	-
Negative	7	7	-	-	-	-	-	-	-

TABLE 8/21

INCIDENCE OF ANTINUCLEAR ANTIBODIES IN SJÖGREN'S SYNDROME

Source	Group	No. of patients	No. of positive sera	"Homogeneous"	"Speckled"	"Nucleolar"
N.I.H. (Washington) series	A (K.C.S. + definite R.A.	18	10 (56%)	8	2	-
	B (K.C.S. + possible R.A.	2	1	-	1	1*
	C (K.C.S. + P.S.S.)	3	2	-	2	-
	D (K.C.S. + myopathy)	3	3	2	1	-
	E (K.C.S. alone)	16	12 (75%)	7	5	5**
	TOTAL	42	28 (66.6%)	17	11	6
Glasgow series		35	20 (57%)	10	10	2***
TOTAL		77	48 (62%)	27	21	8
Controls		77	5 (6.5%)	4	1	-

* This patient's serum also contained "speckled" antibody.
** 2 patients' sera also contained "homogeneous" antibody, and 3 patients' sera also contained "speckled" antibody.
*** One patient's serum also contained "homogeneous" antibody and the other also contained "speckled" antibody.

CODE

K.C.S. = Keratoconjunctivitis sicca
R.A. = Rheumatoid arthritis

TABLE 8/22

ANTINUCLEAR ANTIBODIES IN SJÖGREN'S SYNDROME

Relationship of keratoconjunctivitis sicca and xerostomia to presence of antinuclear antibodies

		Number of patients	Number of patients	
			ANF -ve	ANF +ve
Keratoconjunctivitis sicca	Present	38	12	26
	Absent	4	2	2
Xerostomia	Present	36	13	23
	Absent	6	1	5

TABLE 8/23
SEX INCIDENCE OF ANTINUCLEAR ANTIBODIES

SEX INCIDENCE OF ANTINUCLEAR ANTIBODIES

	Number	Number with antinuclear antibodies
Males	2	2
Females	40	26

TABLE 8/24
"
ANTINUCLEAR ANTIBODIES IN SJOGREN'S SYNDROME

Relationship of parotid gland enlargement to presence of antinuclear antibodies

Parotid gland enlargement	No.	ANF -ve	ANF +ve
Present	22	3	19
Absent	20	10	10

$\chi^2 = 4.893$ $P > 0.05$

TABLE 8/25

ANTINUCLEAR ANTIBODIES IN SJÖGREN'S SYNDROME

Relationship between serum γ globulin levels and presence of antinuclear antibodies in groups A and E

	Serum γ globulin level (g./100 ml.)		
	< 1.4	1.5 - 1.9	> 2.0
Group A	4 (9)	3 (5)	3 (4)
Group E	1 (2)	1 (1)	10 (13)

The total number of patients studied in each group is shown within brackets

TABLE 8/26

ANTINUCLEAR ANTIBODIES IN SJÖGREN'S SYNDROME

Relationship between Bentonite flocculation test (B.F.T.) for rheumatoid factor and titre of antinuclear antibodies

Whole Washington Series

A.N.F.	1/32	B.F.T. 1/64	1/128	1/256	1/512	1/1024	1/2048	1/4096
Neg.	3	1	2	3	1	2	1	1
1/16				HH H	S	H		H
1/64		H		H	 H	H S		HHH S H
1/256		S			S	 S H	HH	HH S
1/1000		SS H	H		 S H	H		H S

H = "Homogeneous" antibody; S = "Speckled" antibody; N = "Nucleolar" antibody

Group A

A.N.F.	B.F.T.							
	1/32	1/64	1/128	1/256	1/512	1/1024	1/2048	1/4096
Neg.	1	1	2	1		2		1
1/16				H		H		
1/64						H		HHH S
1/256							H	
1/1000		S						H

H = "Homogeneous" antibody; S = "Speckled" antibody; N = "Nucleolar" antibody

Group E

A.N.F.	B.F.T.							
	1/32	1/64	1/128	1/256	1/512	1/1024	1/2048	1/4096
Neg.	1			1	1		1	
1/16				H N	S			H
1/64		H			N	S		N
1/256					S	S N	H	H
1/1000			H		S N	H		

H = "Homogenecus" antibody; S = "Speckled" antibody; N = "Nucleolar" antibody

TABLE 8/27

ANTINUCLEAR ANTIBODIES IN SJÖGREN'S SYNDROME

Relationship between sensitised sheep cell agglutination test (S.S.A.T.) for rheumatoid factor and titre of antinuclear antibody

Whole Washington Series

A.N.F.	S.S.A.T.										
	Neg.	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	1/2048	1/4096
Neg.		1	1	1	5		2	1	1		
1/16	H					S	H N	HH			
1/64	H			H		N		H N	H S		HH
1/256						SS	H SS	HH			H
1/1000		H	S	H		S NN		S			

$\chi^2 = 0.07$ $P = 0.8$

H = "Homogeneous" antibody; S = "Speckled" antibody; N = "Nucleolar" antibody

Group A

A.N.F.	S.S.A.T.										
	Neg.	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	1/2048	1/4096
Neg.		1	1		2		1	1	1		
1/16	H							H			
1/64						S		H	H		HH
1/256								H			
1/1000	S			H							

H = "Homogeneous" antibody; S = "Speckled" antibody; N = "Nucleolar" antibody

Group E

A.N.F.	S.S.A.T.										
	Neg.	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	1/2048	1/4096
Neg.				1	2						
1/16						S	H N	H			
1/64	H					N		N	S		
1/256						S	H S	H	N		
1/1000		H				N		S			

H = "Homogeneous" antibody; S = "Speckled" antibody; N = "Nucleolar" antibody

TABLE 8/28

ANTINUCLEAR ANTIBODIES IN SJÖGREN'S SYNDROME

Relationship between tanned cell agglutination reaction (T.R.C.) for antithyroglobulin and antinuclear antibodies

Uncle Washington Series

A.N.F.	T.R.C.										
	Neg.	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	1/2048	1/4096	1/25,000
Neg.	10		1					2			
1/16	HH S N								H	H	
1/64	HHHH S N			H	H				 S N		
1/256	HH SS						H		 S N		H
1/1000	HH SSS N	H							 N	S	

$\chi^2 = 0.66$ $0.5 > P > 0.4$

H = "Homogeneous" antibody; S = "Speckled" antibody; N = "Nucleolar antibody

Group A

A.N.F.	T.R.C. Neg.	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	1/2048	1/4096	1/25,000
Neg.	6		1					1			
1/16	H									H	
1/64	HH S			H	H						
1/256	H										
1/1000	H S										

H = "Homogeneous" antibody; S = "Speckled" antibody; N = "Nucleolar" antibody

Group E

A.N.F.	T.R.C.										
	Neg.	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	1/2048	1/4096	1/25,000
Neg.	3							1			
1/16	H S N								H		
1/64	H N								S N		
1/256	H						H		S N		
1/1000	H S	H							 N		

H = "Homogeneous" antibody; S = "Speckled" antibody; N = "Nucleolar" antibody

TABLE 8/29

ANTINUCLEAR ANTIBODIES IN SJÖGREN'S SYNDROME

Relationship between auto-immune complement fixation reaction (A.I.C.F.) and titre of antinuclear antibodies

Whole Washington Series

A.N.F.	A.I.C.F.				
	Negative	1/40	1/120	1/360	1/1000
Neg.	9			3	1
1/16	H			H	H° S N
1/64	HHHHHH S			S N	N
1/256	H	H	S	S N	H SS
1/1000	H SSS			H N	N

$\chi^2 = 0.2915$ P = 0.6

Group A

A.N.F.	A.I.C.F.				
	Negative	1/40	1/120	1/360	1/1000
Neg.	6			1	
1/16	N				
1/64	HHNH S				
1/256		H			
1/1000	H S				

H = "Homogeneous" antibody; S = "Speckled" antibody; N = "Nucleolar" antibody

Group E

A.N.F.	A.I.C.F.				
	Negative	1/40	1/120	1/360	1/1000
Neg.	1			2	1
1/16				H	H S N
1/64	H			S N	N
1/256	H			S N	H S
1/1000	S			H N	

H = "Homogeneous" antibody; S = "Speckled" antibody; N = "Nucleolar" antibody

TABLE 8/30

INCIDENCE OF PRECIPITATING AUTOANTIBODIES IN SJÖGREN'S SYNDROME

Source	Group	No. of Patients	No. of Positives	Anti-SjD	Anti-SjT	Anti-Lup	Not identified
N.I.H. (Washington) Series	A	18	1	-	-	1	-
	B	2	1	1	1	-	-
	C	3	2	-	-	2	-
	D	3	1	1	1	-	-
	E	16	13	11	6	-	2
	TOTAL	42	18	13	8	3	2
Glasgow Series		35	12	7	4	-	4
Total Series		77	30	20	12	3	6

This data is reproduced with permission from Dr J. R. Anderson who made the experimental observations

TABLE 8/31

SJÖGREN'S SYNDROME

Relationship between antinuclear antibodies and precipitating autoantibodies in whole series

Antinuclear antibodies	Precipitating autoantibodies	
	Negative	Positive
Negative	26	3
Positive	21	26

TABLE 8/32

SJÖGREN'S SYNDROME

Incidence of precipitating autoantibodies in sera containing antinuclear antibodies in whole series

Antinuclear antibody	No.	Precipitating autoantibodies				
		Negative (47)	Anti-SjD (20)	Anti-SjT (12)	Anti-Lup (3)	N.I. (6)
"Homogeneous"	27	17	6	4	-	4
"Speckled"	21	4	11	8	3	2
"Nucleolar"	8	1	7	4	-	-
Negative	29	26	3	-	-	-

N.I. = Not identified

TABLE 8/33

ANTINUCLEAR ANTIBODIES IN RHEUMATOID ARTHRITIS

Previous reports of incidence detected by fluorescent antibody technique

Friou, 1958d	4/42
Calabresi et al., 1959	2/13
Alexander et al., 1960	119/183
Hall et al., 1960	48/130
Bunim, 1961	4/31
Hijmans et al., 1961	43/86
Kratohko, 1961	5/25
Mandema et al., 1961	2/17
Rothfield et al., 1961	5/25
Weir et al., 1961	19/132
Barnes, 1962	17/60
Fennell et al., 1962	3/21
Heaton, 1962	14%
Thompson, 1962	10/68

TABLE 8/34

ANTINUCLEAR ANTIBODIES IN

POLYARTERITIS NODOSA AND DERMATOMYOSITIS

Previous reports of incidence detected by fluorescent antibody technique

	Polyarteritis nodosa	Dermatomyositis
Friou, 1958d	-	1/2
Alexander et al., 1960	3/3	1/1
Hall et al., 1960	1/4	2/3
Kratchko, 1961	0/1	0/4
Mandema et al., 1961	1/1	0/2
Weir et al., 1961	-	2/4
Fennell et al., 1962	0/5	0/1
Thompson, 1962	0/2	0/8

TABLE 8/35

ANTINUCLEAR ANTIBODIES IN PERNICIOUS ANAEMIA

Comparison of incidence in patients and healthy controls matched for age and sex

	No. of Patients	Antinuclear antibodies			
		No. positive	"Homogeneous"	"Speckled"	"Nucleolar"
Pernicious anaemia	50	9 (18%)	9	-	1
Controls	50	3 (6%)	3	-	-

TABLE 8/36

RELATIONSHIP BETWEEN ANTINUCLEAR ANTIBODIES AND OTHER IMMUNOLOGICAL ABNORMALITIES IN PERNICIOUS ANAEMIA

		No. of Patients	Antinuclear antibodies	
			Positive	Negative
Fluorescent antibody test for antibodies to gastric mucosa	+	43	8	35
	-	7	1	6
T.R.C. test for antithyroglobulin	+	12	2	10
	-	38	7	31
Precipitin test for antitissue antibodies	+	7	2	5
	-	43	6	37
Hyland "R.A." test for rheumatoid factor	+	10	2	8
	-	40	7	34

TABLE 8/37

INCIDENCE OF ANTINUCLEAR ANTIBODIES IN MISCELLANEOUS SKIN DISEASES

Disease	No. of patients	No. of positive	Remarks
Generalised scleroderma	3	1	Titre 1/64 "homogeneous"
Morphoea	9	2	Titres 1/16 "homogeneous" 1/16 "speckled"
Nodular vasculitis	5	1	Titre 1/256 "homogeneous" This patient later developed S.L.E.
Cutaneous polyarteritis	8	0	
Allergic vasculitis	1	0	
Rheumatoid arteritis	2	0	
Erythrocyanosis	1	0	
Raynaud's phenomenon	6	0	
Chilblains	7	0	
Weber-Christian panniculitis	1	0	
Wegener's granulomatosis	2	0	
Purpura (undetermined origin)	6	0	
Henoch-Schönlein purpura	3	0	
Idiopathic thrombocytopenic purpura	2	0	
Erythema multiforme	12	0	
Actinic dermatitis	6	0	
Exfoliative dermatitis	3	0	
Drug sensitivity	2	0	
Cholinergic urticaria	1	0	
Lichen myxoedematosus	1	0	

TABLE 8/37 (Continued)

Disease	No. of patients	No. of positive	Remarks
Pityriasis lichenoides varioliformis acuta	1	0	
Jessner's lymphocytic infiltration	1	0	
Degos syndrome	1	0	
Reiter's syndrome	2	0	
Bechet's syndrome	1	0	
Lupus vulgaris	1	0	
Cutaneous sarcoidosis	1	0	
Cryoglobulinaemia	1	0	
Waldenstrom's macroglobulinaemia	1	0	
Polymyositis	1	0	
Familial eosinophilia	1	0	
TOTAL	103	4	

TABLE 8/38

INCIDENCE OF ANTINUCLEAR ANTIBODIES IN VARIOUS DISEASES

Disease	No. of Patients studied	Nuclear staining patterns				Incidence of antinuclear antibodies	H:S ratio
		"Homogeneous"	"Speckled"	"Nucleolar"	"Membranous"		
Systemic lupus erythematosus	80	43	20	2	3	66 (82.5%)	2.1
Discoid lupus erythematosus	137	33	15	1	-	48 (35%)	2.2
Progressive systemic sclerosis	32	17	8	6	-	25 (78%)	2.1
Sjögren's syndrome	77	27	21	8	-	48 (62%)	1.3
Rheumatoid arthritis	103	20	4	-	-	24 (24%)	5.0
Polyarteritis nodosa	9	-	-	-	-	-	-
Dermatomyositis	3	-	-	-	-	-	-
Pernicious anaemia	50	9	-	1	-	9 (18%)	-
Miscellaneous skin diseases	103	4	-	-	-	4 (4%)	-

TABLE 9/1

EFFECT OF MERCAPTO ETHANOL TREATMENT ON SERA REMOVED FROM MOTHERS ON DAY OF CHILDBIRTH

	Fluorescent antibody test for antinuclear antibody						Hyland "R.A." test for rheumatoid factor
	Mrs May. 1st pregnancy		Mrs.May. 2nd pregnancy		Mrs Dal.		Control rheumatoid arthritis serum
	Mother	Baby	Mother	Baby	Mother	Baby	
Treated Mercapto ethanol	+	+	+	+	+	+	-
Treated control buffer	+	+	+	+	+	+	+

TABLE 9/2

RESULTS OF TESTS FOR ANTINUCLEAR ANTIBODIES

IN COLOSTRUM, MILK AND AMNIOTIC FLUID

Date of specimen given in days after childbirth (where appropriate)

Patient	Colostrum	Milk	Amniotic fluid
Mrs May. (1st pregnancy)	.....	negative (12)	.....
Mrs May. (2nd pregnancy)	negative (1, 2 and 3)	negative (14)	negative *
Mrs Dal.	negative (1, 2 and 3)	negative (12)	negative *

* specimens concentrated 10 times by pressure dialysis before testing

TABLE 11/1

COMPARISON OF DIFFERENTIAL COUNTS MADE ON NORMAL BLOOD FILMS (BEC.) STAINED BY "SPECKLED" ANTIBODY AND LEISHMAN METHOD

	STAIN	
	Tra./4	Leishman
Polymorphonuclear leucocytes	232 (67.1%)	252 (64.3%)
Lymphocytes	96 (27.7%)	116 (29.6%)
Monocytes	18 (5.2%)	24 (6.1%)
TOTAL	346	392

Tra./4 = "Speckled" antinuclear serum (Tra.) diluted 1 in 4

TABLE 11/2

COMPARISON OF DIFFERENTIAL COUNTS MADE ON BLOOD FILMS FROM CHRONIC MYELOID LEUKAEMIA (GAL.) STAINED BY "SIECKIED" ANTINUCLEAR ANTIBODY AND LEISHMAN METHOD

	STAIN	
Cell Types	Tra./4	Leishman
Myelocytes	19 (2.3%)	22 (3.0%)
Metamyelocytes	243 (29.4%)	227 (31.4%)
Stab Cells	27 (3.3%)	61 (8.4%)
stained	173 (21.0%)	
Polymorphs unstained	326 (39.5%)	
total	499 (60.5%)	404 (55.8%)
Lymphocytes	37 (4.5%)	10 (1.4%)
TOTAL	825	724

TABLE 11/3

COMPARISON OF DIFFERENTIAL COUNTS MADE ON BLOOD FILMS FROM CHRONIC MYELOID LEUKAEMIA PATIENT (MAR.) STAINED BY "SPECKLED" ANTINUCLEAR ANTIBODY AND LEISHMAN METHOD

	STAIN	
Cell Types	Tra./4	Leishman
Myelocytes	79 (10.0%)	66 (8.2%)
Metamyelocytes	190 (24.1%)	148 (18.5%)
Stab Cells	13 (1.7%)	59 (7.4%)
stained	67 (8.5%)	
Polymorphs unstained	365 (46.4%)	
total	432 (54.9%)	457 (57.1%)
Lymphocytes	73 (9.3%)	70 (8.8%)
TOTAL	787	800

TABLE 11/4

COMPARISON OF DIFFERENTIAL COUNTS MADE ON BLOOD FILMS FROM CHRONIC MYELOID LEUKAEMIA PATIENT (AND.) STAINED BY "SPECKLED" ANTINUCLEAR ANTIBODY AND LEISHMAN METHOD

	STAIN	
Cell Types	Ral./4	Leishman
Myelocytes	48 (5.8%)	53 (6.6%)
Metamyelocytes	365 (44.2%)	342 (42.6%)
Stab Cells	29 (3.5%)	71 (8.8%)
stained	137 (16.6%)	
Polymorphs unstained	199 (24.0%)	
total	336 (40.6%)	332 (41.4%)
Lymphocytes	49 (5.9%)	5 (0.6%)
TOTAL	827	803

Ral./4 = "Speckled" antinuclear serum (Ral.) diluted 1 in 4

TABLE 11/5

DIFFERENTIAL COUNT MADE ON BLOOD FILMS FROM CHRONIC MYELOID LEUKAEMIA PATIENT (RAL.) STAINED BY "SPECKLED" ANTINUCLEAR ANTIBODY

Cell Type	Tra./4
Myelocytes	69 (8.5%)
Metamyelocytes	191 (23.4%)
Stab Cells	49 (6.0%)
Polymorphs stained	318 (39.0%)
Polymorphs unstained	138 (17.0%)
Polymorphs total	456 (56.0%)
Lymphocytes	50 (6.1%)
TOTAL	815

TABLE 12/1

ANTIGENS IN PROTOZOAL NUCLEI

TAXONOMIC CLASS	SPECIES	Nuclear staining	
		Anti-nucleohistone	Anti-DNA
Mastigophora	BLOOD FORMS		
	Trypanosoma gambiense	-	+
	T. rhodesiense	-	+
	T. rhodesiense 200	-	+
	T. cruzi	-	+
	T. congolense	-	+
	T. congolense K 71	-	+
	T. equiperdum	-	+
	T. evansi S.A.K.	-	+
	T. lewisi	-	+
	CULTURES		
	Trypanosoma rhodesiense	-	+
	T. congolense	-	+
	T. lewisi	-	+
	T. cruzi	-	+
	Strigomonas	-	+
	Leishmania donovani	-	+
Sporozoa	Toxoplasma gondii (peritoneal exudate)	-	+
Sarcodina	Amoeba proteus (culture)	Gross autofluorescence	
Ciliophora	Paramecium sp. (culture)	+	+
	Peranema sp. (culture)	+	+

TABLE 12/2

CONTROL OF SPECIFICITY OF STAINING BY ANTI-DNA AND ANTI-NUCLEOHISTONE ANTINUCLEAR SERA

	Staining Serum									
	Anti-NH					Anti-DNA				
	Pre-Digestion		Absorption			Pre-Digestion		Absorption		
Organism	DNase	RNase	DNA	RNA	NH	DNase	RNase	DNA	RNA	NH
T. gambiense (blood film)	•••	•••	•••	•••	•••	-	+	-	+	-
T. rhodesiense (culture)	•••	•••	•••	•••	•••	-	+	-	+	-
Strigomonas (culture)	•••	•••	•••	•••	•••	-	+	-	+	-
Toxoplasma gondii	•••	•••	•••	•••	•••	-	+	-	+	-
Peranema (culture)	-	+	+	+	-	-	+	-	+	-

NH = Nucleohistone

APPENDIX 1/1

PREPARATION OF FLUORESCEIN-CONJUGATED ANTI-HUMAN-γ-GLOBULIN

1. Preparation of human-γ-globulin for use as antigen

This was prepared by column chromatography (Peterson and Sober, 1960) from pooled normal human serum using either diethylaminoethyl cellulose (DEAE cellulose, Whatman) or DEAE Sephadex (Pharmacia, Uppsala) as absorbents. Columns were prepared by the method of Peterson and Sober (1956), charged with serum which had been previously dialysed against 0.02M-phosphate buffer (pH 6.3) and eluted with the same buffer. The elute was concentrated by pressure dialysis (Mill Hill Method, Humphrey, 1960) until it contained approximately 50 mg. protein/ml. The protein content was determined by the optical density at 280 mμ measured on a Unicam SP 500 spectrophotometer. This protein fraction migrated as a single band with the mobility of γ globulin on filter paper electrophoresis.

2. Immunisation procedure

Four buck Chinchilla rabbits were immunised by the Mill Hill Schedule (Humphrey, 1960).

(a) Preparation of Freund's adjuvent mixture Anhydrous lanolin (5 ml.) was placed in a mortar (temperature 50°C). The human γ globulin solution (5 ml.) was added, 1 ml. at a time, and worked in until it formed a uniform thick yellow creamy paste. Liquid paraffin (20 ml.) containing 0.4 ml. killed tubercle bacilli was then

added and worked into a uniform cream. This was stored at 4°C and warmed to 37°C before use.

(b) Preparation of alum-precipitated antigen 4.6 ml. 1.0M-$NaHCO_3$ was added dropwise to 10 ml. human γ globulin solution (50 mg./ml.) with constant shaking. 10 ml. aqueous potassium alum was then added and the mixture left at 4°C overnight. After centrifugation, the deposit was suspended in 25 ml. 0.067M phosphate buffer (pH 6.7) and merthiolate (1/10,000) added as preservative.

(c) Injection schedule 1.0 ml. adjuvant mixture was injected intramuscularly into the right leg and 1.0 ml. into the left leg one week later. Intravenous injections of the alum-precipitated human γ globulin were started on the 6th week and 0.05, 0.1, 0.1 and 0.2 ml. volumes were administered on alternate days. Serious anaphylactic reactions were not encountered. The animals were bled on the 4th day after the last injection. The antibody content of the immune sera was estimated by quantitative precipitation curves against purified human γ globulin using the method recommended by Kabat and Meyer (1946). The immune rabbit sera used in these investigations contained 8.6, 7.7, 6.9 and 4.8 mg. anti-human-γ-globulin/ml. These sera each produced only two lines in gel diffusion studies against whole human serum.

3. Preparation of fluorescent conjugate

The method used for chemical combination of fluorescein to antibody

protein in this investigation was suitable both for experimentally produced anti-human-γ-globulin sera used in the sandwich technique and also for human antinuclear sera.

In the conjugation reaction, dye was allowed to react with a fraction of serum rich in γ globulin in preference to whole serum as

(a) this avoids labelling of non-antibody proteins which may cause staining of tissue by non-immunological reactions of the type described previously (Book, 1962b)

(b) the dye compound used in the conjugation reaction is very expensive so that the use of the γ globulin fraction results in considerable economy.

The serum was fractionated by salting out with sodium sulphate by a modification of Kekwick's method:

(i) Serum treated with 18% Na_2SO_4 at room temperature for 6 hr.

(ii) Centrifuge at 3,000 r.p.m. for 30 min. at room temperature.

(iii) Discard supernatant.

(iv) Note volume of precipitate and dissolve in minimum volume of distilled water noting the volume required.

(v) Add Na_2SO_4 to make solution 16%, assuming that precipitate contained 18% Na_2SO_4.

(vi) Leave at room temperature for 18 hr.

(vii) Centrifuge at 3,000 r.p.m. for 30 min. at room temperature and discard supernatant.

The final precipitate is rich in γ globulin. It was dissolved in the minimum volume of distilled water and dialysed in Visking tubing against four changes of 0.15M-NaCl (pH 7.4) over a period of 24 hr in order to remove the sodium sulphate completely. Such exhaustive dialysis was probably unnecessary but was undertaken because Kaufman and Cherry (1961) have shown that contamination with ammonium sulphate interfered with conjugation. The authors considered that interference was caused by the ammonium cation rather than by the sulphate anion, but they were unable to prove this. The protein content of the dialysed γ globulin fraction was calculated from the optical density of the solution at 280 mμ using a conversion factor (0.75) to give the concentration in the mg./ml. (Humphrey, 1960).

In the earlier experiments the conjugation reaction was undertaken following the method of Riggs et al. (1958) carefully. 10 ml. 0.15M-NaCl, 3 ml. carbonate-bicarbonate buffer (pH 9.0, 0.5M) and 2 ml. reagent grade acetone were placed in a conical flask fitted with a mechanical stirrer and cooled with an acetone-dry ice freezing mixture until ice crystals started to form. 10 ml. of globulin fraction containing 25 mg. protein/ml. was added and stirring started. Thereafter the flask was cooled periodically in the freezing mixture so that a few ice crystals were constantly present inside the flask. 1.5 ml. reagent grade acetone containing 12.5 mg. amorphous fluorescein isothiocyanate (FITC) (Nutritional Biochemical Co.) was

then added slowly. Thereafter the reaction mixture was stirred overnight at 4°C, run through an anion-exchange column, "Deacidite FF" (Permutit Co.) and dialysed against several changes of 0.15M-NaCl (pH 7.4) at 4°C for 2 days or longer until there was no fluorescent material in the dialysate.

These earlier conjugates were never entirely satisfactory as a large quantity of protein was lost by precipitation during the final dialysis. Furthermore, the conjugates caused intense non-specific staining, which had to be removed by absorption with acetone dried rat and guinea pig liver powders, often requiring two absorptions with 100 mg. powder/ml. conjugate.

In later experiments, the conjugation procedure was modified in three ways

(a) Smaller proportion of FITC In a careful study of the conjugation technique, Goldstein et al. (1961) have shown that the coupling ratio of 50 mg. FITC/g. protein (recommended by Riggs et al., 1958) was excessive and they advised a ratio of 6 to 8 mg. crystalline FITC/g. protein. Highly purified crystalline FITC was not available for this work and so a compromise was made in an attempt to prevent overlabelling of the protein by the use of a coupling ratio of 20 mg. amorphous FITC/g. protein.

(b) Improved cooling system When acetone-dry ice was used to cool the reaction flask, control of temperature was erratic, so that there was frequent freezing and thawing of the reaction mixture and

as this was taking place at pH 9.0 in the presence of acetone, this must have caused considerable protein denaturation. There would also be unpredictable variations in the concentrations of the different reactants. As Sokol et al. (1962) have claimed that the reaction temperature is not an important factor in conjugation, it was decided to cool the reactant flask with a mixture of salt and ice (temp. - 5°C) so that freezing of the contents was avoided.

(c) Improved purification after conjugation completed Dialysis was a time consuming process during which protein denaturation could continue. This has now been eliminated by the introduction of column chromatography with "Sephadex", a material which shows marked molecular seiving properties. This material has been shown by several groups of workers to be very suitable for the removal of unconjugated dye, alkaline buffer and acetone (Curtain, 1961; Goldstein et al., 1961; Killander et al., 1961; Zwaan and Van Dam, 1961; Gordon et al., 1962), but Fothergill and Nairn (1961) prefer activated charcoal.

"Sephadex" treatment was adopted in this investigation because it undertakes simultaneously the adjustment of pH and the removal of unconjugated dye and acetone. The technique will be described in detail.

"Sephadex G 50" medium grade (Pharmacia, Uppsala, Sweden) was suspended in barbitone buffered 0.15M-NaCl and allowed to settle for 3 to 5 minutes. The supernatant containing "fines" was discarded

and the sedimented material resuspended in a new batch of saline. The settling process was repeated until all the finely divided material was removed. The coarser material was then suspended in saline as a "slurry" and immediately poured into a glass tube which was constricted at one end, where the lumen was loosely plugged with glass wool. Sufficient "Sephadex" was used so that the height of the column after settling would be approximately 10 x diameter. Such columns were perfused with approximately 10 times their volume of saline taking care not to disturb the upper surface of the column, which was protected by a disc of filter paper. Saline was allowed to drain from the bottom until the meniscus reached the surface of the column and then the conjugate solution was applied cautiously avoiding either drying or disturbance of the upper surface of the column. The characteristics of any column can be calculated from its physical properties but this is time consuming and so in this investigation the columns were intentionally underloaded with conjugate preparation equivalent to only quarter of the column volume. When the meniscus of the conjugated protein preparation reached the upper surface of the column, buffered saline was added gently to fill the glass tube and so aid perfusion by raising the hydrostatic pressure. As the preparation passed down the column, two bands develop and progressively separate. The more rapidly moving band had a light yellow colour and contained the dye conjugated protein, whereas the slower intensely yellow narrower band contained

unconjugated dye, buffer and acetone. The column often showed a local translucency as the second band was passing through. Collection started when the first band appeared in fairly high concentration in the effluent and was stopped when its colour started to become less intense. Relatively little conjugated protein was lost as the leading and trailing boundaries were fairly sharp. During passage through the column, the conjugate was usually diluted only twofold. If the conjugated serum was of high titre the effluent from the column was often sufficiently concentrated for immediate use and the dilution minimized non-specific staining. When patients' sera were conjugated directly, the effluent required concentration by pressure dialysis.

Conjugated globulin preparations were stored frozen at - 20°C in small tubes, each containing 0.5 - 1.0 ml. so that frequent freezing and thawing would be avoided. This is particularly important as conjugated proteins are much more readily denatured than the corresponding native proteins.

APPENDIX 8/1

The patient, a married woman aged 41 years, was diagnosed in 1956 as suffering from subacute lupus erythematosus without evidence of systemic involvement. She was treated with mepacrine and the skin rash improved. In March 1961, she was admitted to the Western Infirmary, Glasgow with clinical evidence of systemic involvement (fever, chest pain and extension of rash). This was confirmed by laboratory tests: E.S.R. (Westergren) 48 mm. in first hour, haemoglobin 9.5 g./100 ml., W.B.C. 2,500/cu.mm., L. E. cell test strongly positive, blood urea 63 mg./100 ml. and a reversed plasma A/G ratio. She was treated systemically with steroids for 7 months and she showed a dramatic clinical improvement and the L. E. cell test became negative. In October 1962, she had a further exacerbation with a strongly positive L. E. cell test and this time her urine contained three parts Esbach of albumin. She was again treated with steroids but the disease remained active and L. E. cells persisted while the Esbach reading for albumin in the urine fluctuated between 2 parts and 8 parts; she developed hypertension under steroid therapy (blood pressure 160/110 mm.Hg).

She was admitted to the Western Infirmary, Glasgow on 2nd July 1963 with extensive lupus erythematosus of face, arms, hands and trunk, a strongly positive L. E. cell test and a mild pancytopenia. Fluorescent antibody test for antinuclear antibodies showed "speckled" antibody (titre 1/1000) and "homogeneous" antibody (titre 1/64).

Precipitin test showed strong anti-tissue antibody which could not be classified in the scheme proposed by Anderson et al. (1961b, 1962). Since this woman was in incipient renal failure and was not responding to high doses of steroids, thymectomy was performed by Mr K. Fraser on 8th July 1963. Histological examination of the thymus showed that it was atrophic; no germinal centres or plasma cells were seen. Five days later, her rash was fading although the L. E. cell test was still positive. On the 13th post-operative day, the urine showed only $\frac{1}{8}$ part Esbach and the steroid therapy was discontinued. She was discharged on 31st July 1963, virtually free from rash and with only minimal albuminuria.

One week later she developed painful swellings in one leg. These were diagnosed as "thrombophlebitis" by her general practitioner who prescribed Terramycin and Butazolidin for 8 days. Thereafter she developed extensive diffuse erythema with scattered purpura over the entire body. The appearances suggested that this was a sensitization reaction and not lupus erythematosus. She was readmitted on 4th September 1963 and found to have mild anaemia (Hb 10 g./100 ml.), leucopenia (W.B.C. 2,800/cu.mm.) and nine parts Esbach albumin in the urine. Steroid therapy was restarted and the rash faded rapidly and the albuminuria rapidly fell to $\frac{1}{4}$ part Esbach. She was discharged on 25th September 1963 with maintenance dosage of steroids. Since then the skin rash has cleared and only a trace of albumin has been found in the urine. She was last seen on 27th

October 1963.

Fluorescent antibody tests for antinuclear antibodies and precipitin tests for anti-tissue antibodies were strongly positive before the operation and these antibodies have remained unchanged in titre and type during the post-operative period.

My thanks are due to Dr J. A. Milne for permission to reproduce this abstract of the patient's case history.

APPENDIX 8/2

Lupus erythematosus with erythema multiforme-like lesions and characteristic immunological abnormalities - A NEW SYNDROME

Case 1. This woman, now aged 48 years, has had lupus vulgaris of the lower lateral aspect of the right calf since the age of 8. At the age of 28 years, this was treated with carbon dioxide snow and two weeks later she developed two raised painful areas on the right leg above the lateral malleolus and these were diagnosed as discoid lupus erythematosus. These lesions have since subsided and reappeared intermittently. At the age of 35 years, the lateral aspect of the feet began to show bluish red erythema considered to be lupus erythematosus. Biopsy when she was 40 years of age confirmed the diagnosis of lupus vulgaris and discoid lupus erythematosus in the lesions. The E.S.R. (Westergren) was 49 mm. in one hour, haemoglobin 13.8g./100 ml. and the white cell count 5,400/cu.mm. L. E. cells were not demonstrated and X-ray examination of the chest was normal. Treatment with Sterogyl, 600,000 units by mouth twice a week, was started and the lupus vulgaris slowly improved. Over the next few years she had some discoid lesions on the face and right thumb but she also developed ringed lesions on the legs which consisted of tender, hot and itchy reddish rings up to 5 cm. in diameter, the edges of which tended to blister. These lasted a few weeks at a time and resembled a toxic erythema.

In July 1962, at 48 years, she had an exacerbation in which discoid lesions on the ears and plaques on the back of the hands were associated with bright red erythema and one ringed lesion on the front of the chest. She had been on antimalarials for six years but at this stage prednisone 5 mg. t.d.s. was added with rapid improvement in the lupus erythematosus lesions although the erythrocyanosis and erythema of the feet persisted. She was known to be sensitive to sulphonamides, penicillin and possibly isonicotinic hydrazide, but at no time was any consistent cause found for her toxic erythema. Results of laboratory investigations are summarised in Table 8/16.

Case 2. This woman, now aged 72 years, has had chilblains since the age of 50. At the age of 56 she developed itchy red patches on the nose, both cheeks and in front of the ears, two months after an attack of "shingles" of the right wrist. She had exacerbations of the lesions on her face every summer for the next five years. Examination on admission to hospital at the age of 61 years showed a raised dusky erythema of the nose, cheeks, chin and neck with erythematous patches on the extensor aspects of the forearms, dorsum of the hands, lower third of the legs and dorsum of the feet, the larger lesions forming rings. There was no lymphadenopathy, splenomegaly or other clinical evidence of systemic disease.

Over the following eight years she had episodes of toxic lesions mainly on the limbs and although a drug cause was suspected no

consistent factor could be incriminated. The lesions frequently took the form of rings, sometimes with marked vesication, necrosis and ulceration. They usually occurred on the limbs, especially the arms, but also sometimes on the face and the neck. Oral steroids did not materially influence their progress or prevent their recurrence.

Histology of one of the ringed lesions showed a vesicle in the stratum corneum and, in parts, the stratum malphigii; it contained an eosinophilic coagulum and was lined by degenerate eosinophilic cells. At the margins of the vesicle the uninvolved epidermis showed infiltration by degenerate leucocytes, and in other areas the whole thickness of the epidermis was necrotic. The dermis showed patchy perivascular cellular infiltration, mainly lymphocytic. Some leucocytes showed karyorrhexis but there was no haematoxylinophilic material or fibrinoid necrosis.

In 1960 she was found to have pernicious anaemia with subacute combined degeneration and then has been treated with Vitamin B_{12}. L. E. cells have never been demonstrated, and the results of laboratory investigations in 1960 are shown in Table 8/16.

Case 3. A woman, now aged 34 years, developed typical discoid lupus erythematosus insidiously mainly in a butterfly distribution on the nose and cheeks and also on the forehead and temples at the age of 28. The lesions were aggravated by sunshine. She had had Raynaud's phenomenon, poor peripheral circulation and chilblains for

many years. She was treated with chloroquin sulphate 200 mg. b. d. and the rash cleared in four months.

At 32 years, when two months pregnant, she developed annular erythematous lesions on the arms and legs. These lesions were up to about 5 cm. in diameter, slightly tender and resembled erythema multiforme. She had not had any infection and she was not taking any drugs. The toes and dorsum of the feet showed a chilblain-like dusky erythema. General examination revealed no other abnormal signs. The results of laboratory investigations are shown in Table 8/16.

Her baby was born in March 1961 and when seen in December 1961, her clinical state was unchanged. The laboratory investigations were repeated and the results were essentially unchanged but six months later her serum globulin had risen to 4.5g./100 ml. and the Rose-Waaler test was positive with a titre of 1 in 1024.

Case 4. A married woman, now aged 31 years, developed erythematous lesions, aggravated by sunshine, on cheeks and nose at the age of 26. A year later red blotches appeared on the sides and back of the legs. Examination at the age of 28 revealed dark, red, indurated lesions which tended to be softer in the centre and were up to 3.0 cm. in diameter. She was considered to have discoid lupus erythematosus of the face. The lesions on the legs were diagnosed as erythema induratum and she was treated with intramuscular streptomycin and oral isoniazide; she was also given oral chloroquine for her face lesions.

All the lesions cleared, but three months later, when she was six weeks pregnant, Professor J. T. Ingram described an eruption like erythema multiforme with nummular and ringed bright red raised lesions on the arms, backs of the fingers and on the lower legs. Two months later the lesions on the cheeks and nose were still present but the ringed lesions on the limbs had disappeared. Laboratory investigations in June 1959 when she was six months pregnant gave the results shown in Table 8/16.

She was delivered of a normal female child in September 1957. Throughout the following two years she continued to have lupus erythematosus on her face, with episodes of ringed lesions on the legs and persistent chilblains.

APPENDIX 9/1

Mrs Nay. A 28 year old married woman had an influenza-like illness in December 1954, at the age of 20, with fever, vomiting, generalised aching pains, and slight cough. A rash on the face developed the same day as she was given sulphonamides. Because of this, the treatment was changed to penicillin. The rash persisted, and within 2 weeks it had spread to the trunk and arms. At this time the erythrocyte sedimentation rate varied from 40 to 60 mm. in 1 hour (Westergren), the haemoglobin was 11.0g./100 ml., W.B.C. 2,600/cu.mm., platelets 127,000/cu.mm., and serum globulin 3.0 g./100 ml. The urine contained albumin but the blood urea was only 27 mg./100 ml. X-ray examination of the chest was normal, and the Wassermann reaction was negative. L. E. cells were not found. In February 1955, there was amenorrhoea and the rash and fever persisted. Treatment with corticotrophin was started, and by the next month her condition had improved considerably.

In April 1955, her joints became stiff and her hair fell out. She had a further febrile episode in June, when she was readmitted to hospital with erythema of the face and finger tips. The E.S.R. was still increased and leucopenia persisted, but she again responded to corticotrophin and she was given a course of oxytetracycline for a urinary infection. She remained well until 1958, when she had a sore throat followed by malaise, joint pains, rash, and lymph-adenopathy. This exacerbation settled with prednisone, but she had

a further episode of fever, joint pains, and lymphadenopathy, and an apical systolic murmur was heard in December 1960. She was treated with prednisone, 60 mg. daily, and once again her condition improved.

First Pregnancy In December 1961, she was 24 weeks' pregnant and feeling very well on 5 mg. of prednisone a day. There were no abnormal findings on examination apart from a slight dusky appearance of the face. Her blood pressure was 110/70 mm. Hg. The results of investigations were as follows: E.S.R. 14 mm. in 1 hr (Westergren), L. E. cells were demonstrated in the peripheral blood, serum proteins 6.7 g. per 100 ml. (albumin 4.1 g., globulin 2.5 g.), serum electrophoresis normal, "homogeneous" type of antinuclear factor present in a titre of 1/256, haemoglobin 10.85 g./100 ml., white cell count 8,400 per cu. mm., platelets 259,000/cu.mm., Wassermann reaction and Coombs test negative, cold agglutinins and cryoglobulins were not demonstrated.

She remained well, and the investigations were unchanged, when she was delivered normally at term of a male child in March 1962. The dose of prednisone was temporarily raised to 20 mg. on the day of delivery. Apart from slight erythema of the face, the patient was very well after delivery, and when she was seen 6 months later there were no abnormal signs.

The baby showed no clinical abnormality at any time in the first 6 months of life. Subsequently, both mother and baby remained symptoms free. The mother continued on a small maintenance dose of

5 mg. prednisone a day.

Second Pregnancy When seen on 19th January 1963, she was 14 weeks' pregnant. She remained well on the maintenance dose of prednisone and the pregnancy was uneventful. A normal female child was delivered on 13th July 1963. The patient and baby were seen weekly and on 18th September 1963 both were well. Investigations carried out on the mother during this period were as follows:-

Date	E.S.R. (mm./hr)	L.E. cells	Serum Protein (g./100 ml.)	Alb.	Glob.	Serum Electro-phoresis	Rose Waaler Test	A.N.F.
9.1.63	22	-				N	+	
27.2.63	14		5.7	3.2	2.5	N		
22.5.63	34	+				N		256 H
12.6.63	37	-	5.8	3.2	2.6	N		1000 H
3.7.63	63	+	5.3	2.8	2.5		-	1000 H
18.9.63	4	+	6.3	3.8	2.5	N		256 H

On 9th January 1963 the Coombs test and W.R. were negative and cold agglutinins and cryoglobulins were not demonstrated.

Hb. 14.0g./100 ml., W.B.C. 9,800/cu.mm., platelets 365,000/cu.mm.

APPENDIX 9/2

Mrs. Dal. - now aged 27, has always had a poor peripheral circulation with chilblains during her teens and Raynaud's phenomenon of the hands and feet for many years. In 1956, at the age of 20, she developed a butterfly erythematous rash on her face together with lupus erythematosus lesions on the backs of the fingers, and nail fold telangiectasia. There was also some erythema of the inner side of the thighs. In 1958, she developed a right sided pleural effusion with anaemia (Hb. 69%), raised E.S.R. (28 mm. in one hour, Westergren), hyperglobulinaemia (globulin 4.7 g./100 ml.) and L.E. cell preparations were positive. This episode settled with prednisone treatment.

In 1959 she had joint pains in the fingers and dexamethasone was substituted for prednisone. In 1960 she developed psoriasis of the scalp. Spontaneous bruising of the lower legs was noticed in 1961, and as it was thought that this might have been caused by dexamethasone, she was treated with prednisone and later triamcinolone. In 1962 she had ulcers on the right ankle and on the front of the right leg but general examination was negative. The blood pressure was 110/70 mm./Hg. Hess' test was negative. Investigations gave the following results:- E.S.R. 15 mm. in one hour (Westergren), Serum protein 7.0% (albumin 4.7%, globulin 2.3%), Electrophoresis - normal, Haemoglobin 13.6 g./100 ml., W.B.C. 4,500/cu. mm., platelets 147,000/cu.mm., W.R., Coombs test, D.A.T., Cryoglobulins and cold agglutinins - negative, L. E. cell test negative, Liver

function tests normal, X-ray examination of chest and electrocardiograph normal. "Homogeneous" antinuclear antibody was present in a titre of 1:256 and antimolecular antibody in a titre of 1:64.

When seen in November 1962, she was 8 weeks' pregnant. The pregnancy was uneventful. She was delivered of a normal child on 14th April 1963, and both mother and baby remained well over the next 6 months.

Investigations

Date	E.S.R. (mm./hr)	Serum Protein (g./100 ml.)	Alb. (g./100 ml.)
28. 5.62	15	7.0	4.7
28.11.62	47	8.1	5.5
30. 1.63	46		
27. 2.63	7	5.7	3.3
10. 4.63	49		
9. 5.63			
21. 5.63			

Date	Glob. (g./100 ml.)	L. E. cells	D.A.T.	A.N.F. (reciprocal)
28. 5.62	2.3	-	-	256 H
28.11.62	2.6		-	64 H
30. 1.63				256 H
27. 2.63	2.4 N	+	-	64 H
10. 4.63		+	-	256 H
9. 5.63				256 H
21. 5.63				256 H

APPENDIX 9/3

Technique for determination of the resistance of antinuclear antibody to degradation by mercapto-ethanol

1. 1 part of serum was treated with 15 parts 0.2M mercapto-ethanol in phosphate buffer (pH 7.4) for 18 hours at $4^{o}C$.
2. The mixture was then dialysed against 3 changes of large volumes of 0.02M iodoacetic acid in phosphate buffer (pH 7.4) for 24 hours.
3. The efficacy of mercapto-ethanol treatment was controlled by treating a rheumatoid arthritis serum with high titre rheumatoid factor simultaneously.

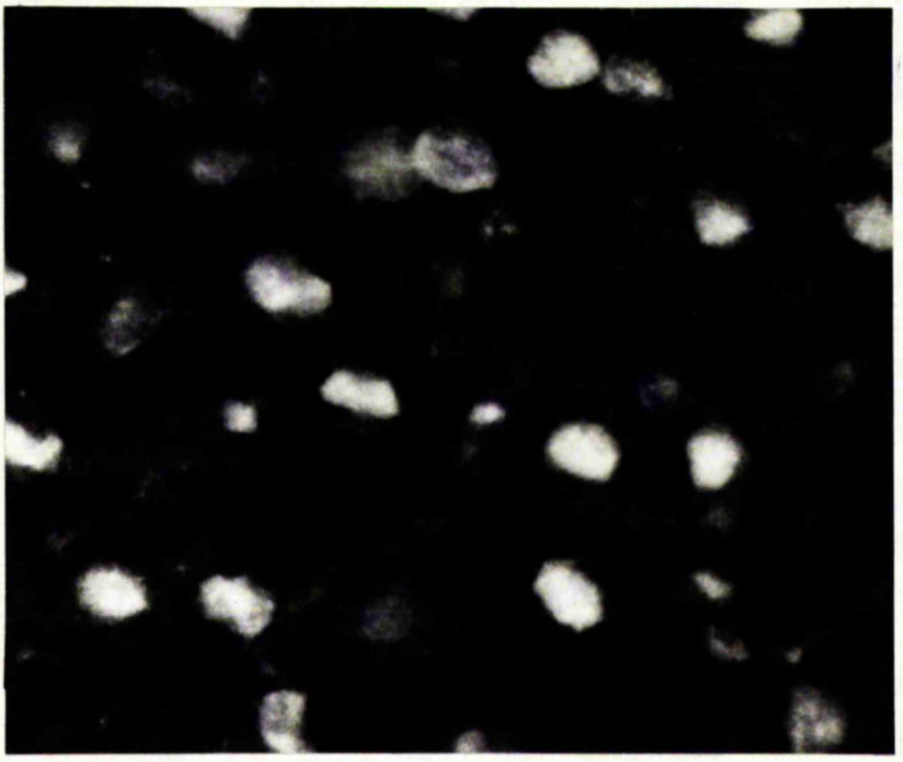


Fig. 1/1 "Homogeneous" pattern of nuclear staining. Fresh frozen section of rat liver stained with serum Dun. x775.

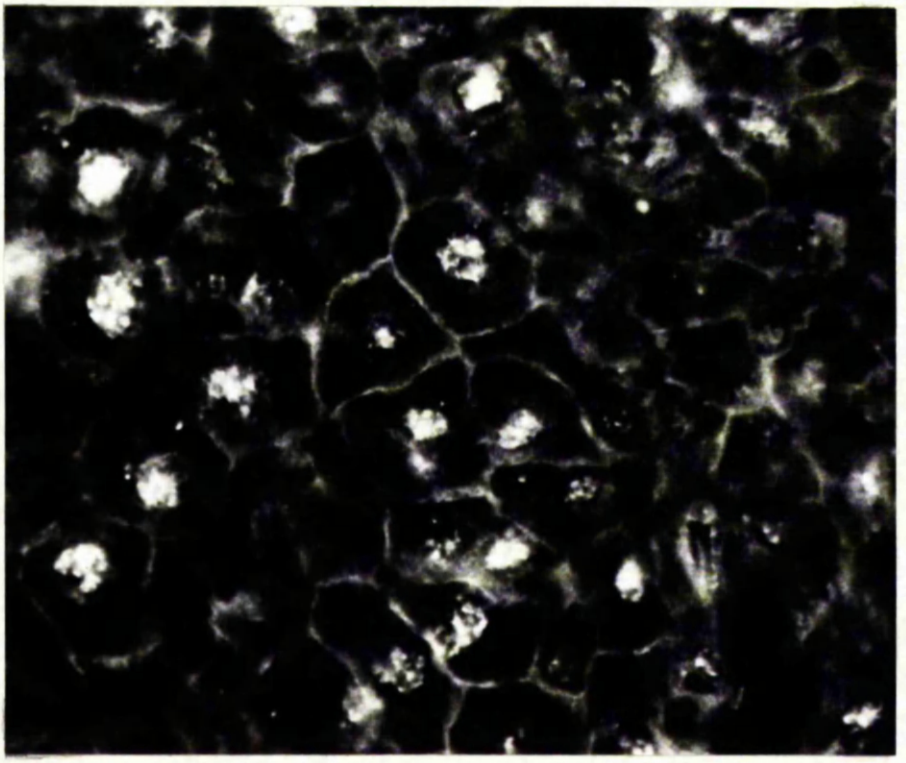


Fig. 1/2 "Speckled" pattern of nuclear staining. Fresh frozen section of rat liver stained with serum Tra. x775.

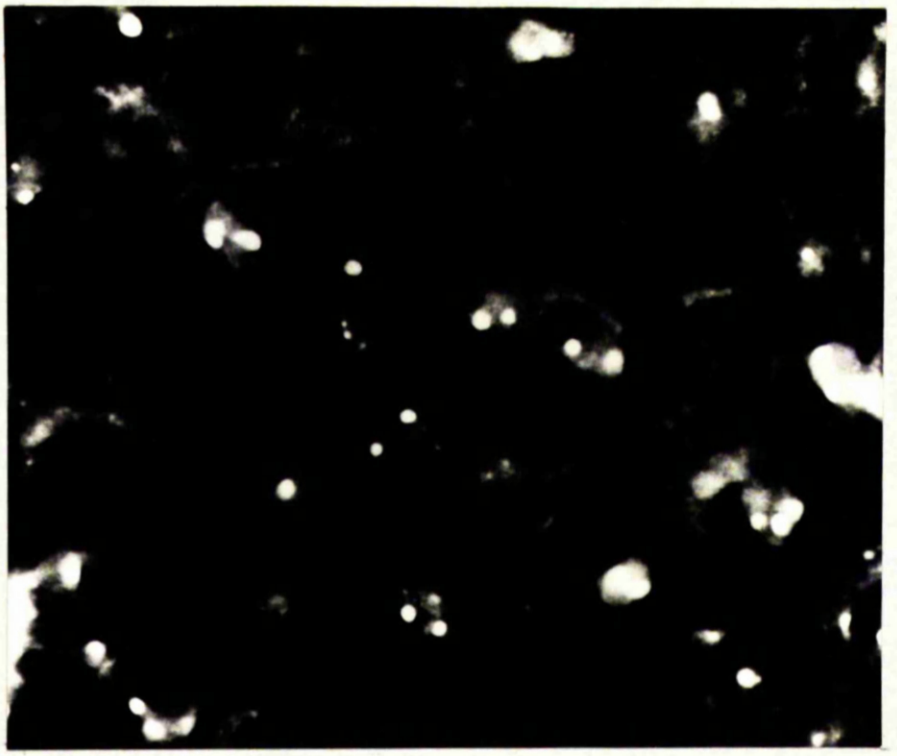


Fig. 1/3 "Nucleolar" pattern of nuclear staining. Fresh frozen section of rat liver stained with serum Mui. x775.

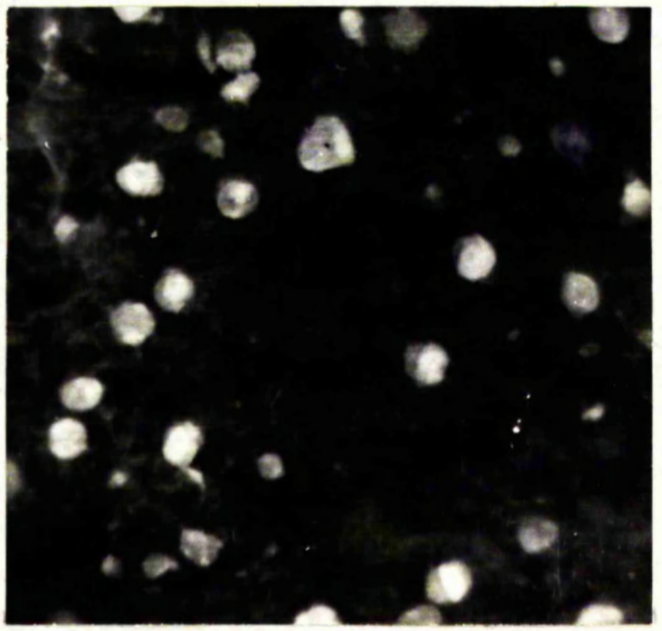


Fig. 1/4 "Membranous" pattern of nuclear staining. Fresh frozen section of rat liver stained with serum McDou. x775.

Fig. 3/1 Isolated mouse liver nuclei stained with "speckled" antinuclear serum Tra. x1000.

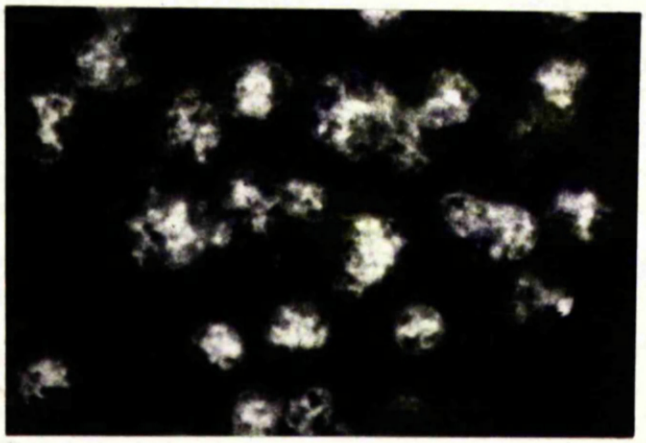
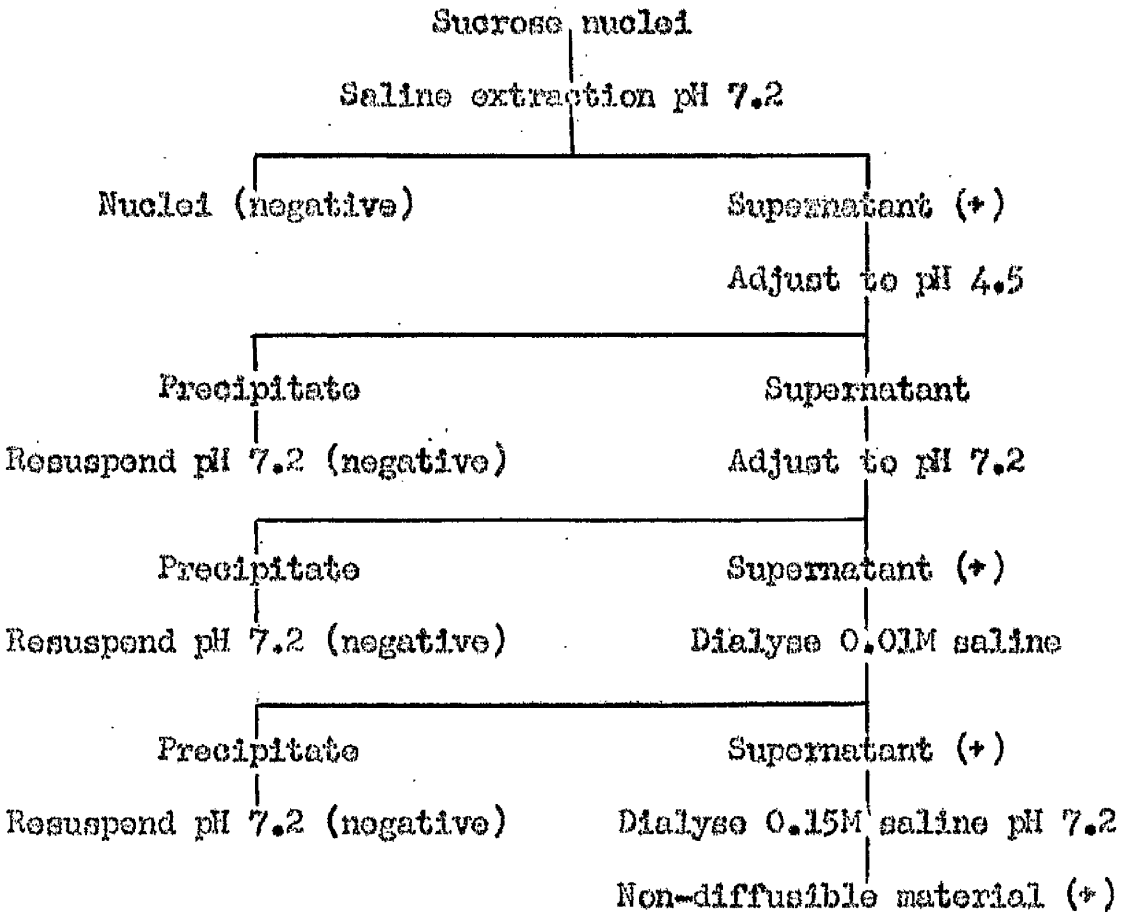


Fig. 3/2 Isolated mouse liver nuclei stained with "homogeneous" antinuclear serum Dun. x800.

Fig. 3/3 Isolated mouse liver nuclei stained with normal serum Bec. x1000.

FIGURE 3/4

SCHEME OF PURIFICATION OF THE "SPECKLED" ANTIGEN



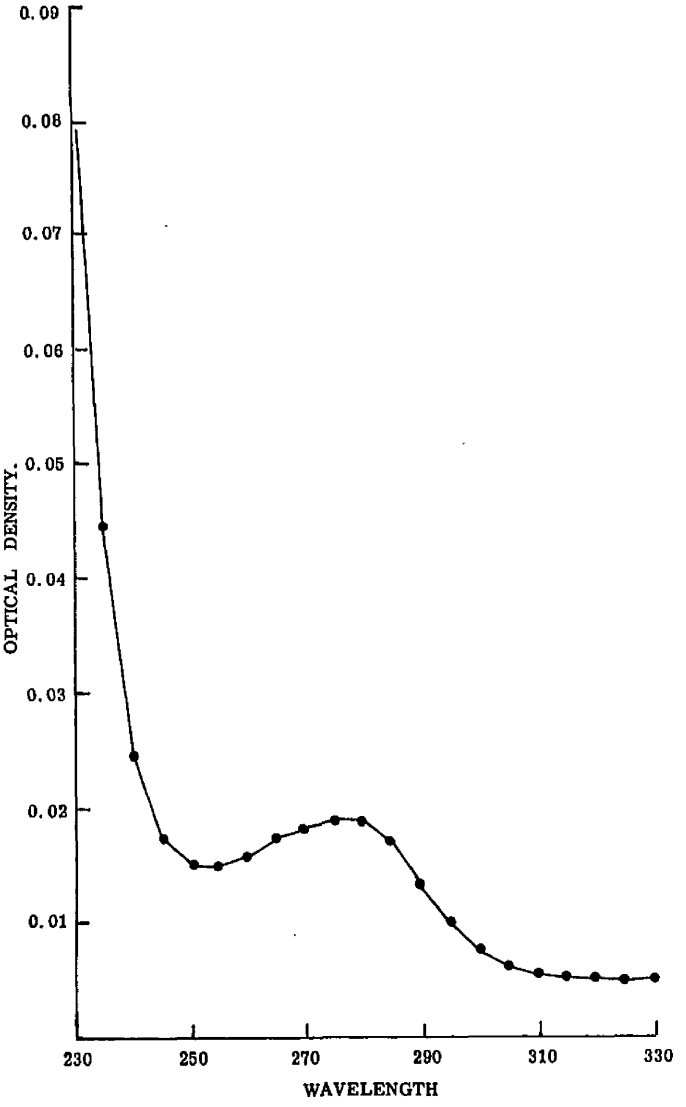


Fig. 3/5 Ultraviolet absorption spectrum of a purified preparation of the "speckled" nuclear antigen.

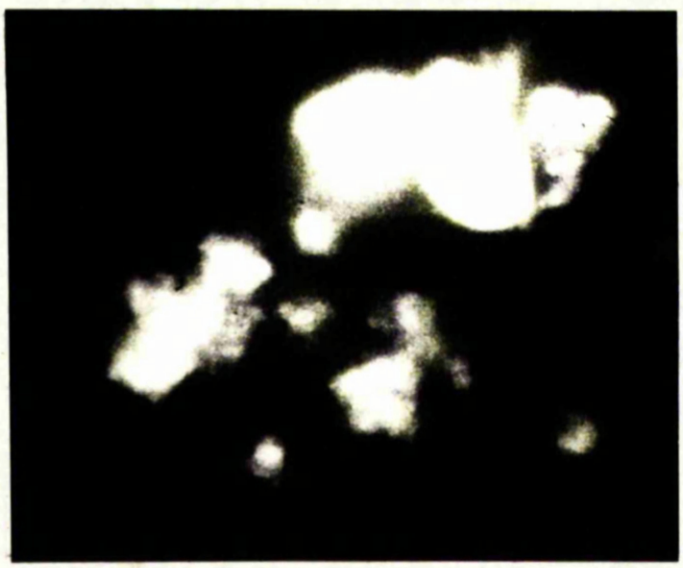


Fig. 4/1 Isolated rat liver nuclei disintegrated by ultrasonication and stained with antinucleolar serum Mui. Many nucleoli are aggregated but a few are free and can be recognised by their characteristic rounded shape.

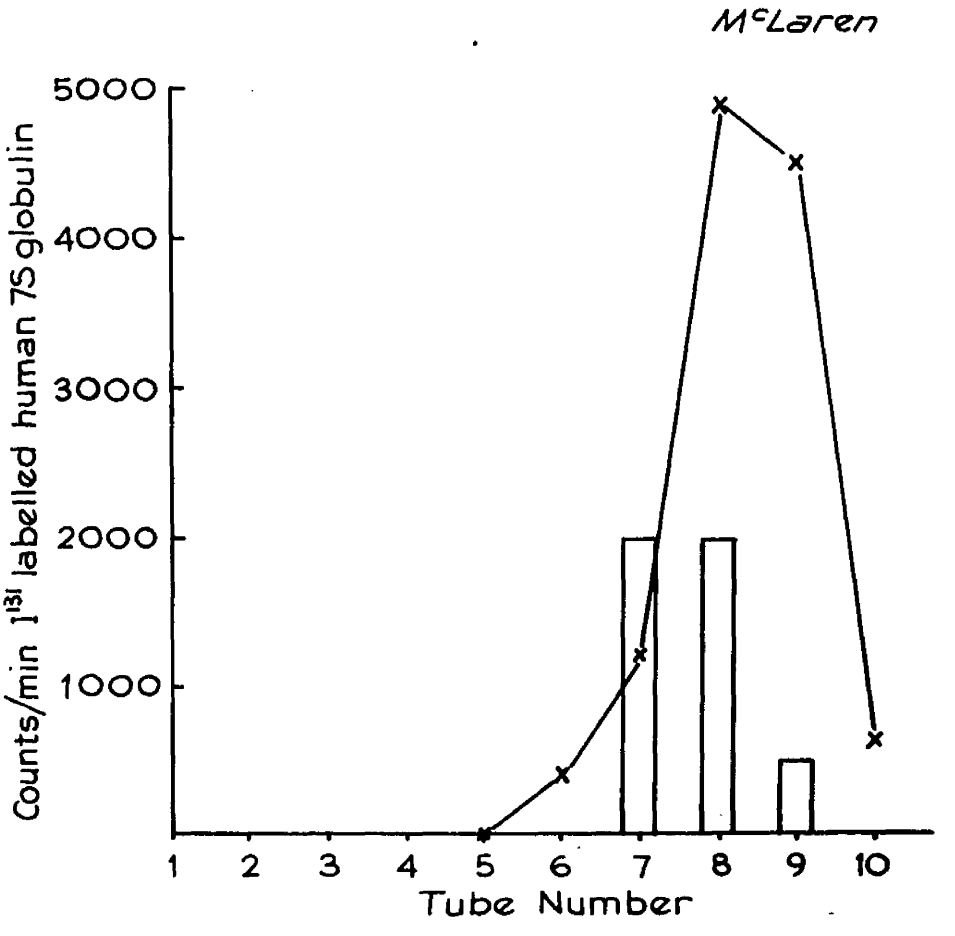


Fig. 6/1 Ultracentrifugation of "homogeneous" antinuclear serum McLar. in sucrose density gradient with marker ^{131}I labelled normal 7.0S globulin.

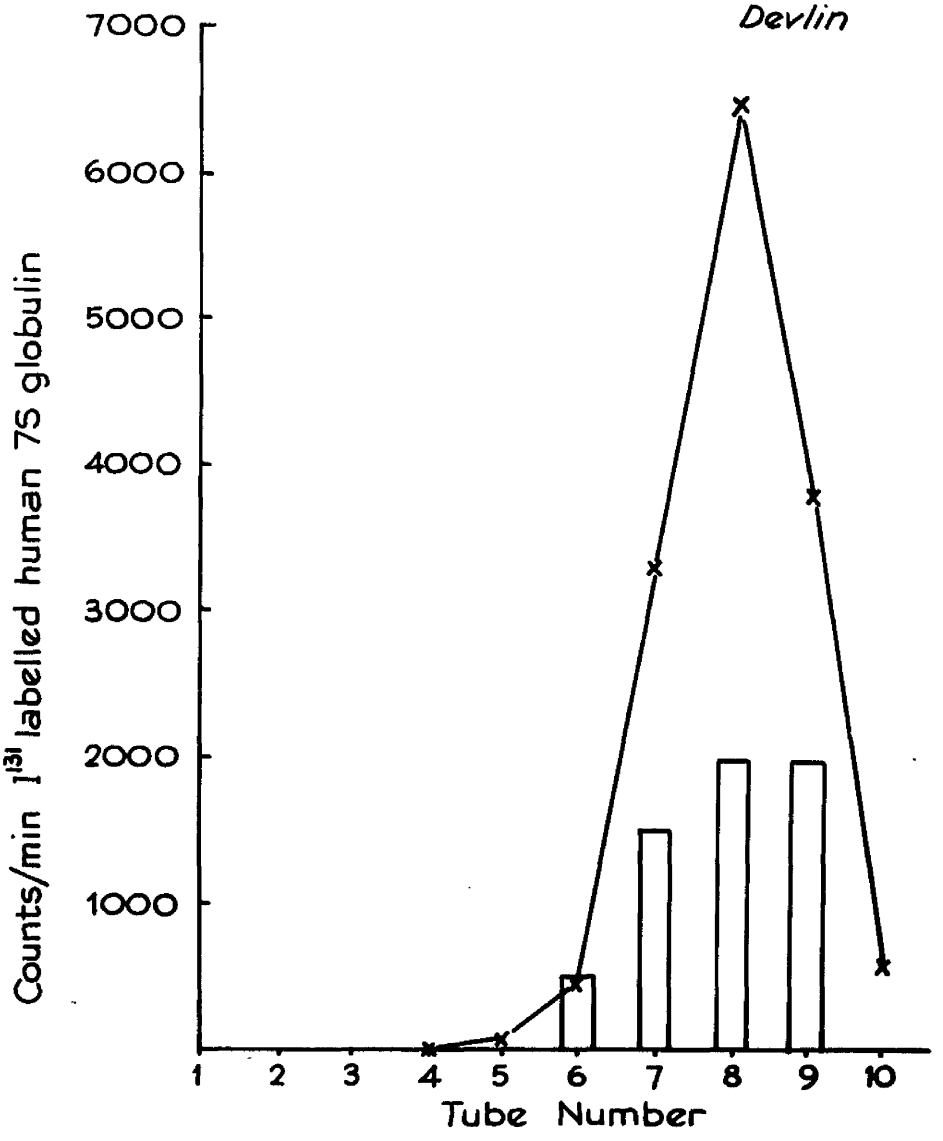


Fig. 6/2 Ultracentrifugation of "speckled" antinuclear serum Dev.

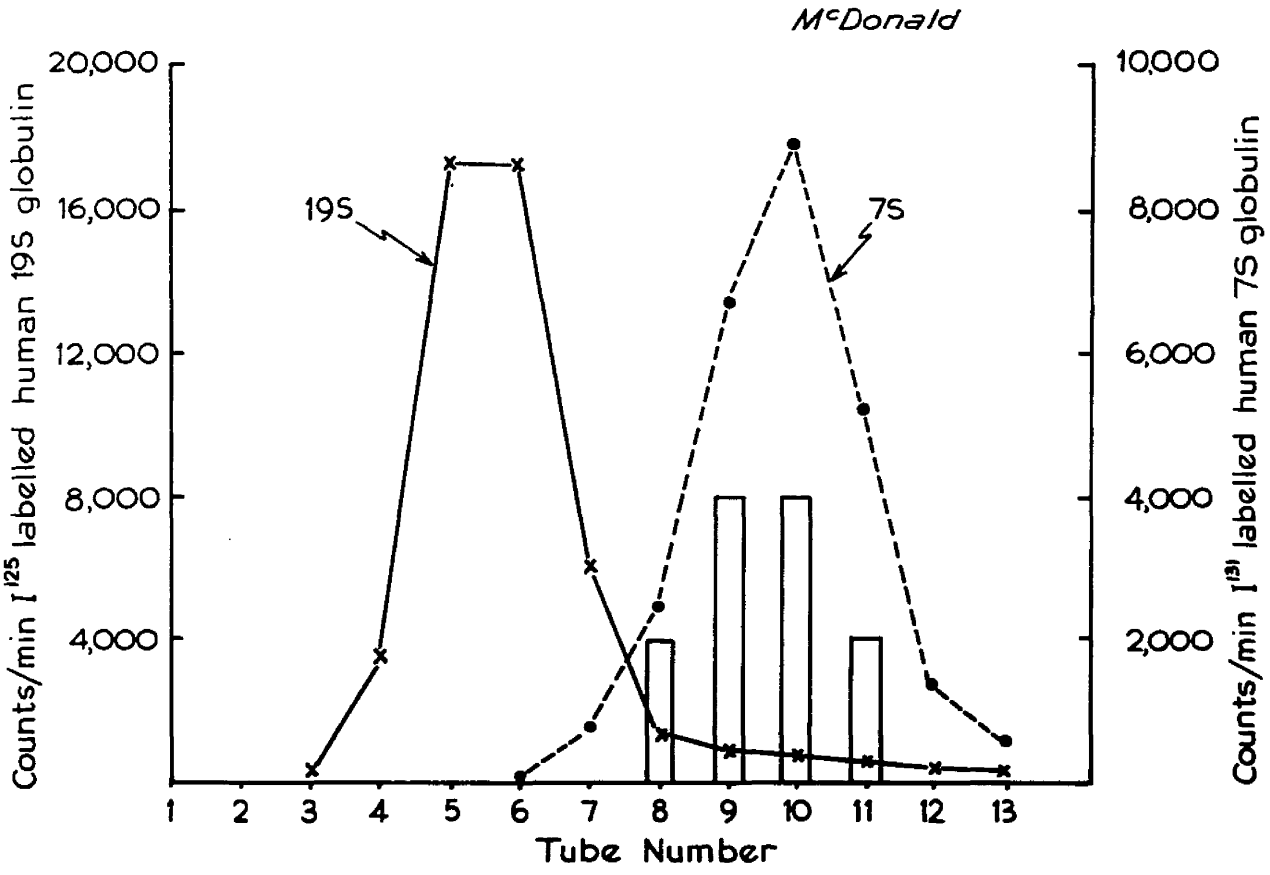


Fig. 6/3 Ultracentrifugation of "speckled" antinuclear serum McDon.

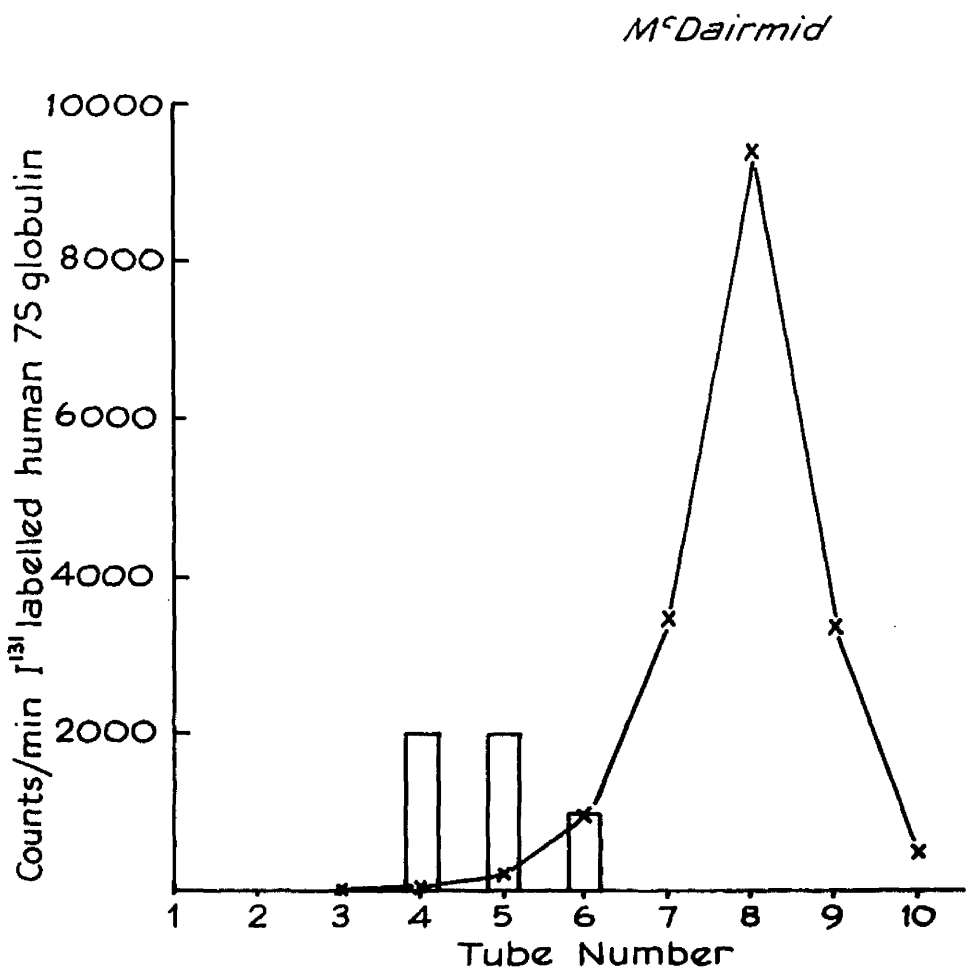


Fig. 6/4 Ultracentrifugation of "homogeneous" antinuclear serum McDai.

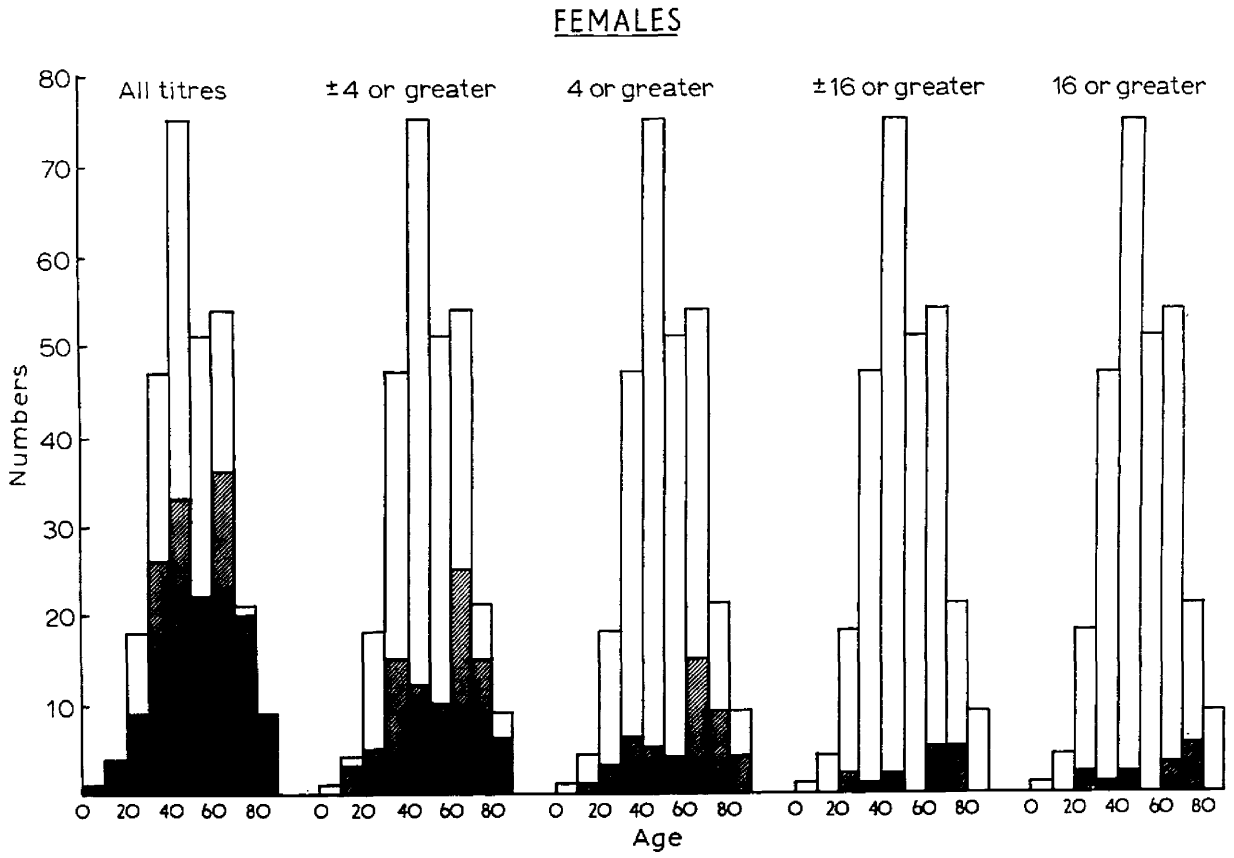


Fig. 7/1 Incidence of antinuclear antibodies in blood grouping sera from 280 consecutive female patients not known to be suffering from autoimmune disease. At each level of sensitivity the positive sera are represented by solid bars in the histograms.

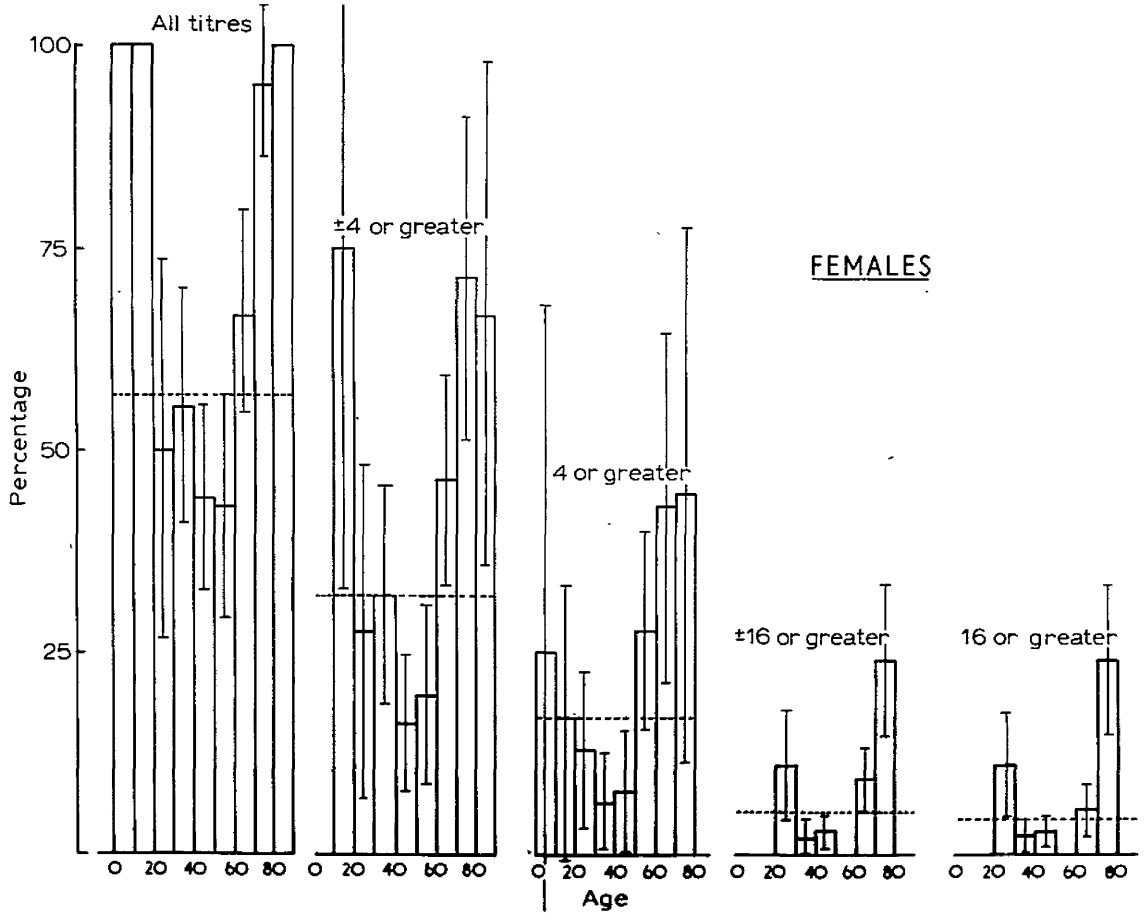


Fig. 7/2 Percentage incidence of antinuclear antibodies in female. The limits of 2 standard deviations are indicated for each age group.

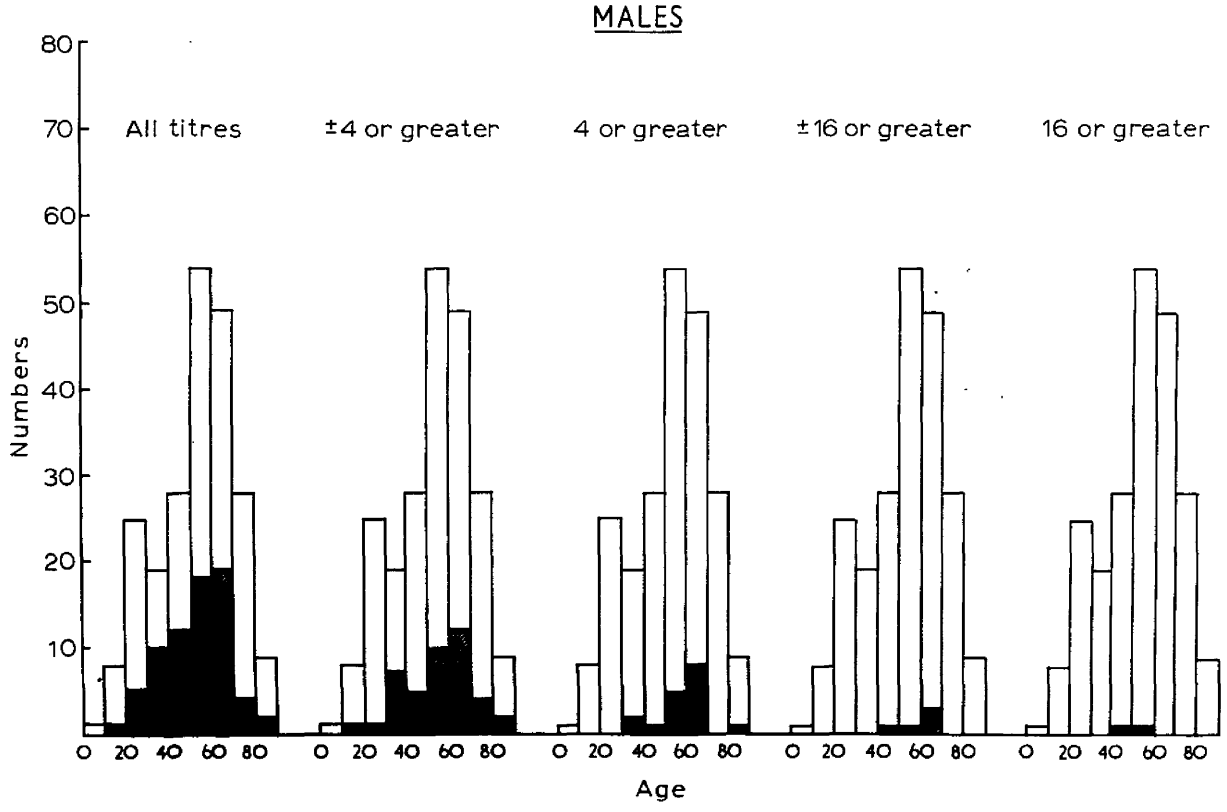


Fig. 7/3 Incidence of antinuclear antibodies in blood grouping sera from 220 consecutive male patients, not known to be suffering from autoimmune disease. At each level of sensitivity the positive sera are represented by solid bars in the histograms.

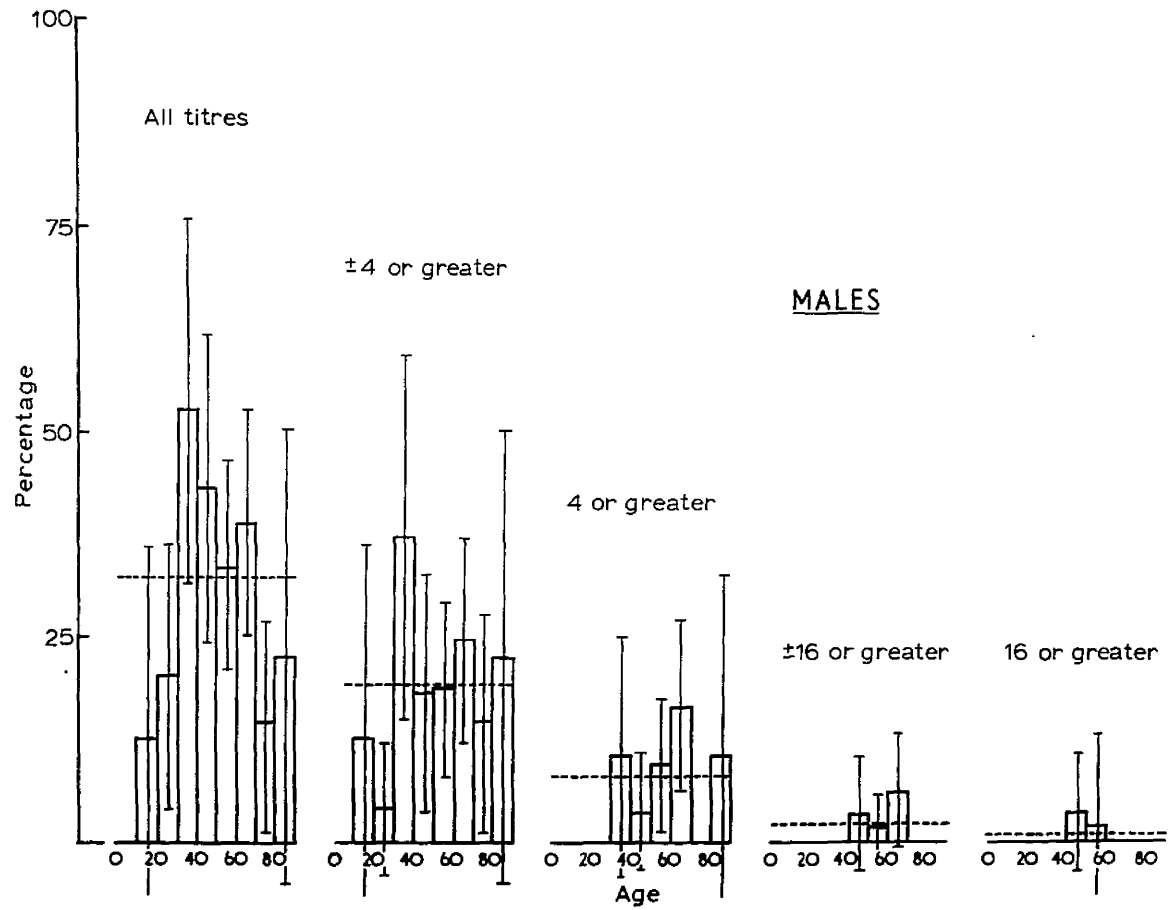


Fig. 7/4 Percentage incidence of antinuclear antibodies in males. The limits of 2 standard deviations are indicated for each age group.

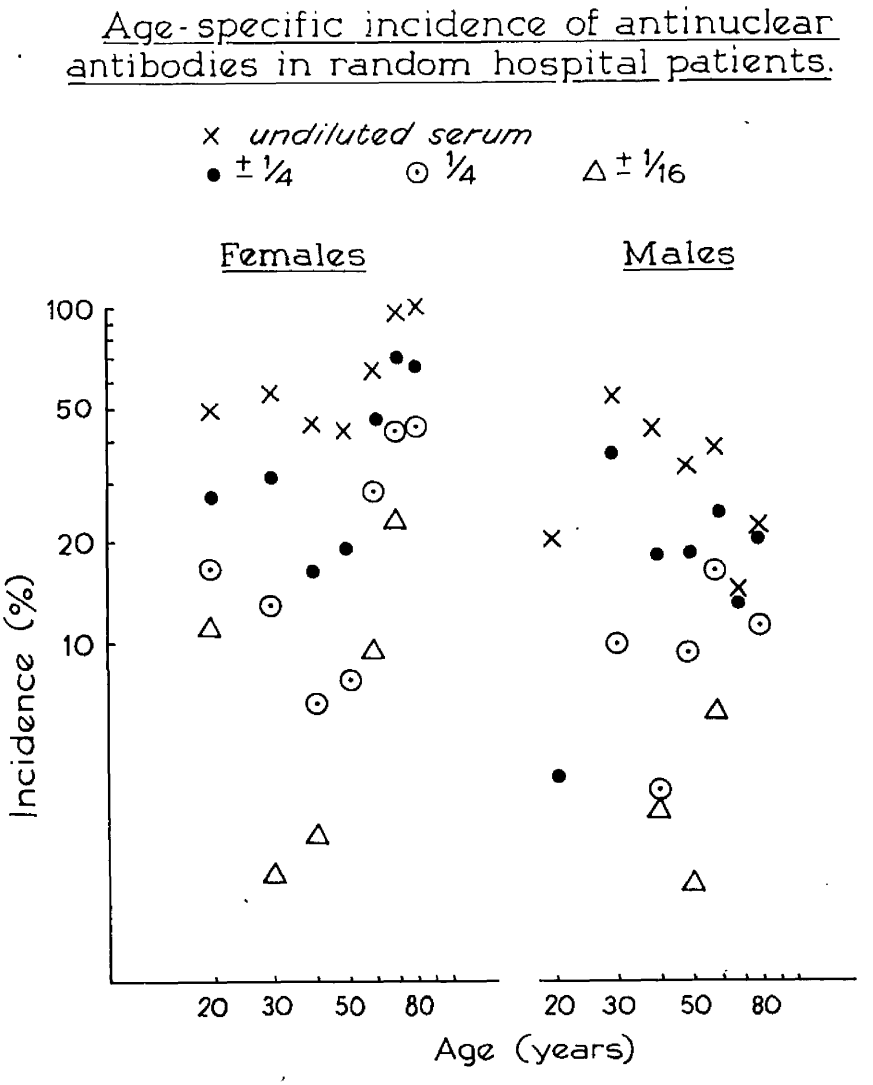


Fig. 7/5 Age-specific incidence of antinuclear antibodies in random hospital patients (in whom autoimmunity was not suspected) plotted on a double logarithmic scale.

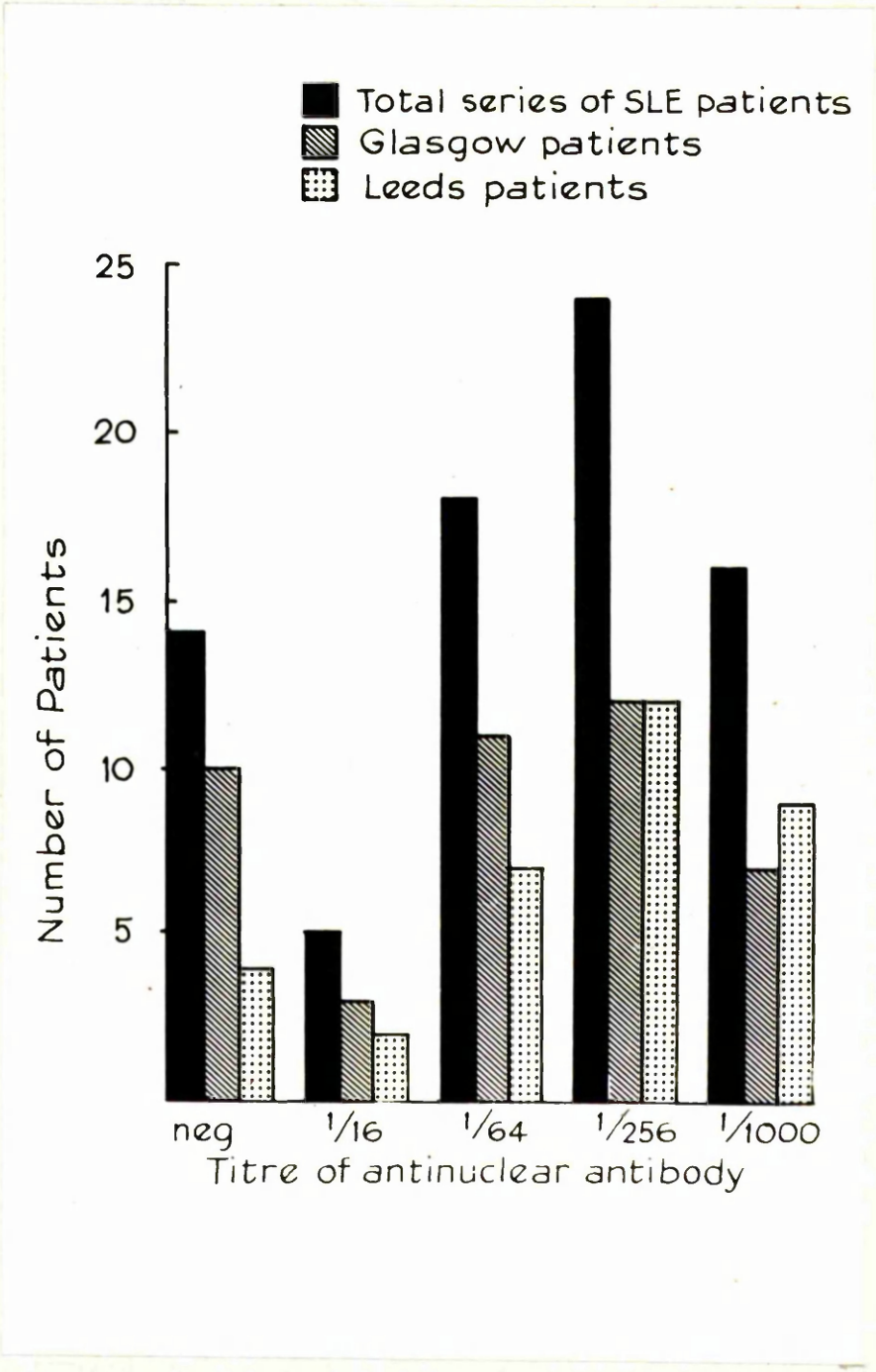


Fig. 8/1 Titres of antimuclear antibodies (all types) in systemic lupus erythematosus.

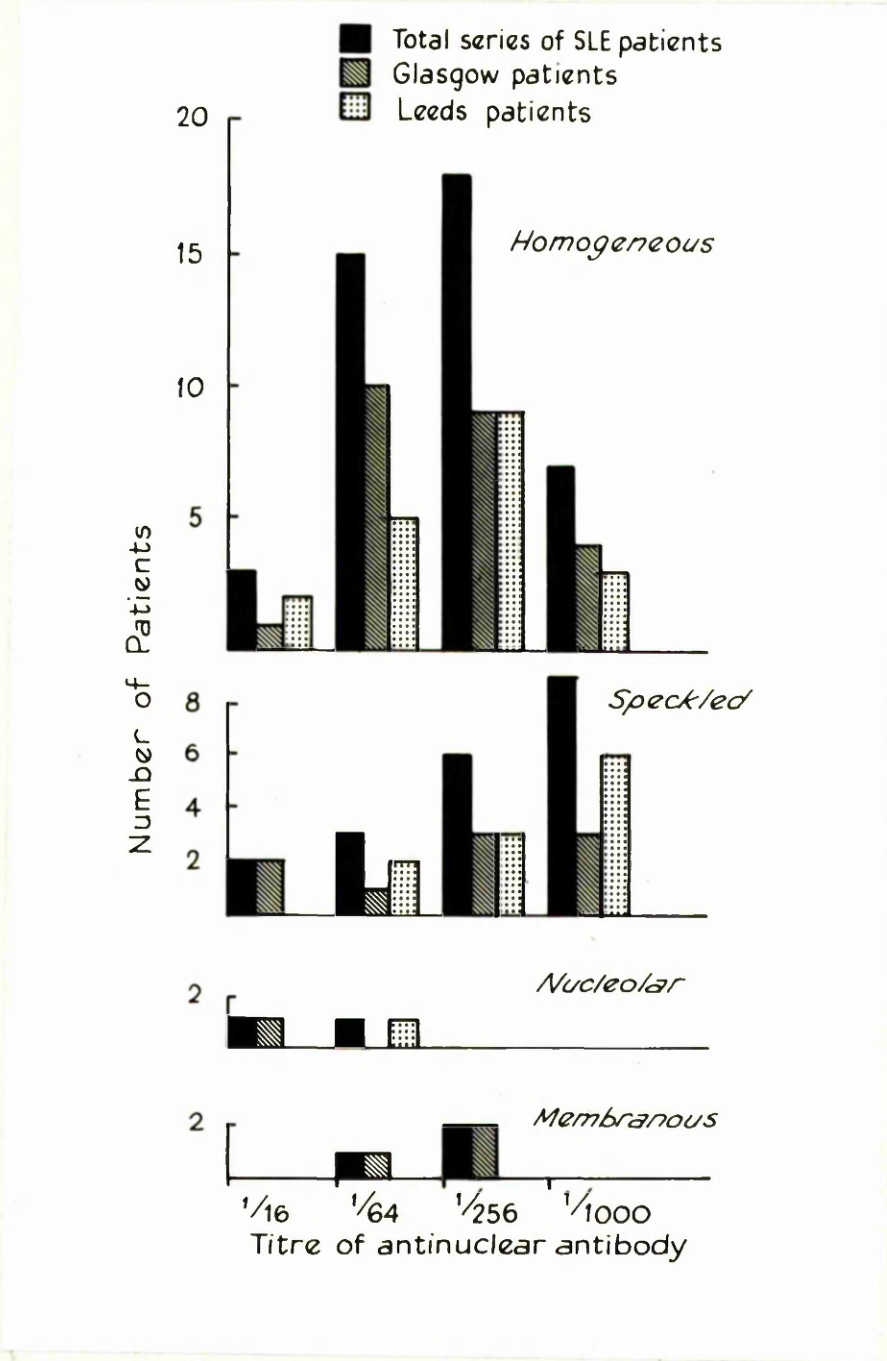


Fig. 8/2 Titres of different systems of antinuclear antibodies in systemic lupus erythematosus.

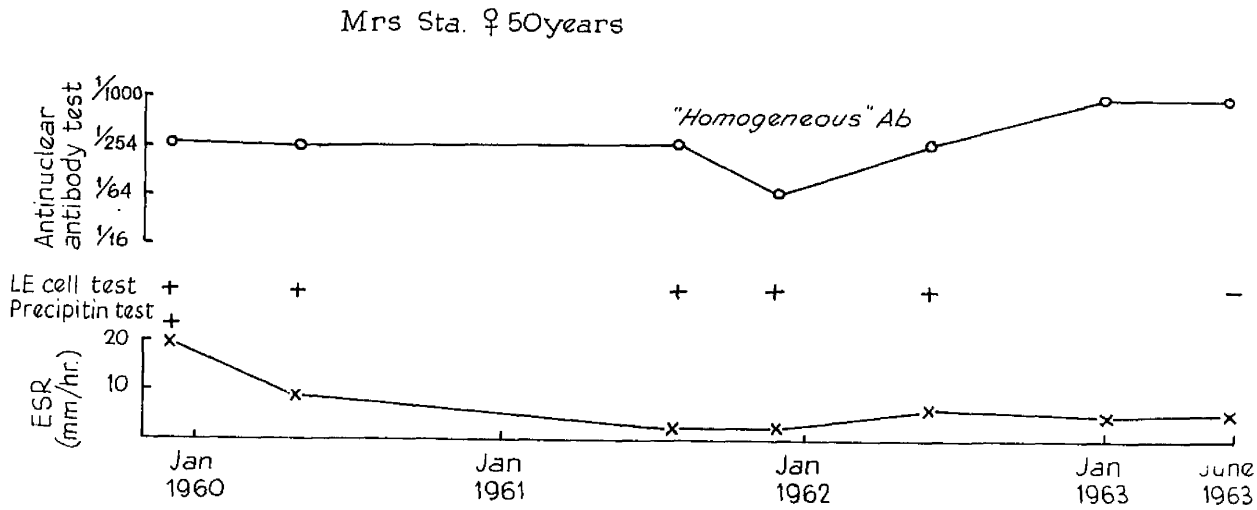


Fig. 8/3 Serial studies on systemic lupus erythematosus patient (Mrs Sta.)

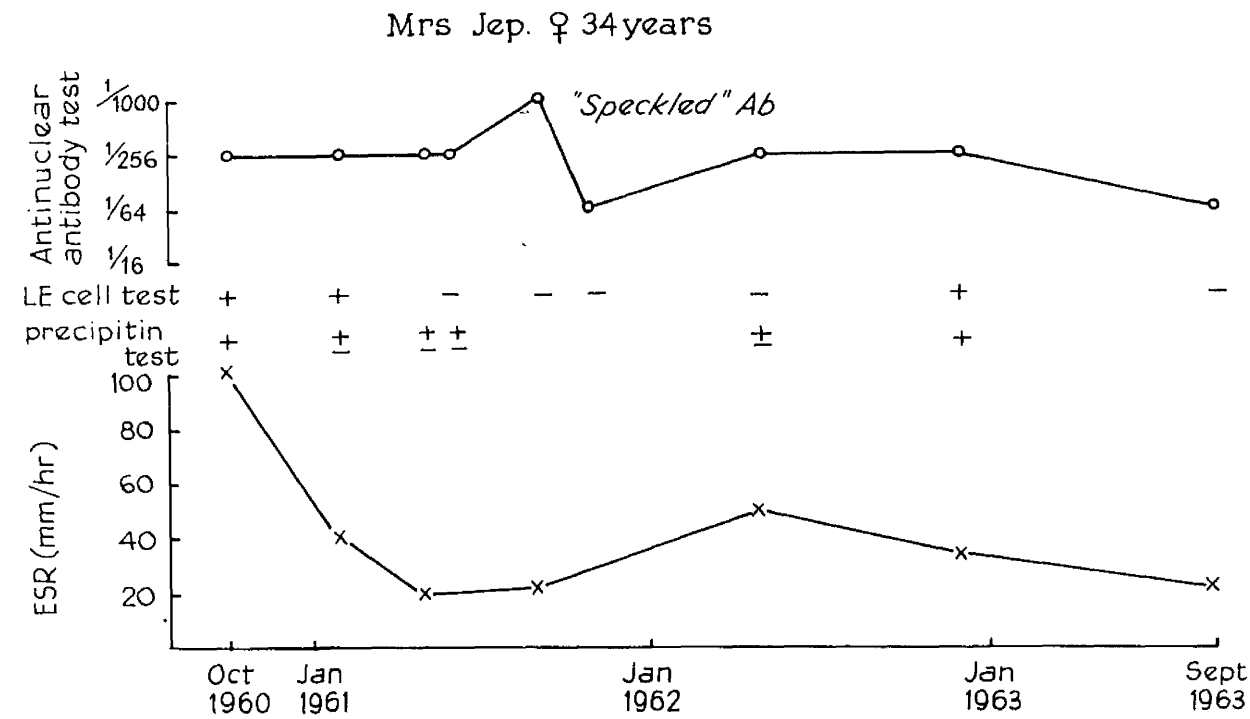


Fig. 8/4 Serial studies on systemic lupus erythematosus patient (Mrs Jep.)

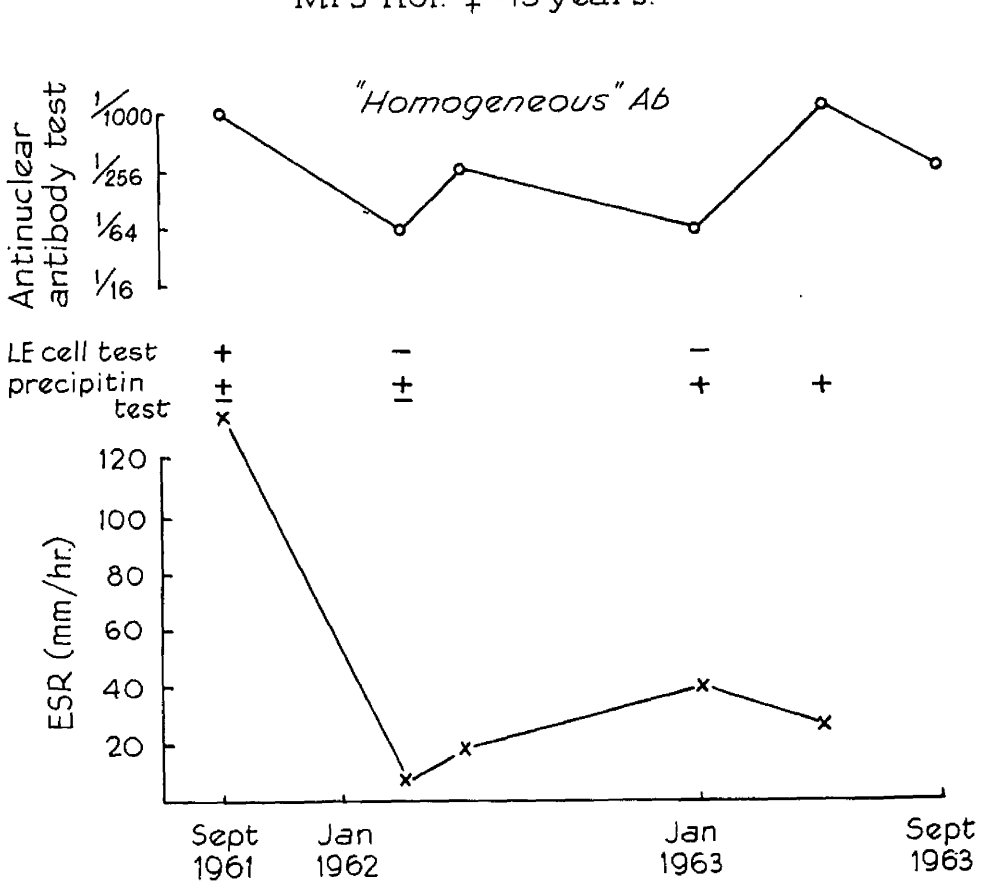


Fig. 8/5 Serial studies on systemic lupus erythematosus patient (Mrs Hol.)

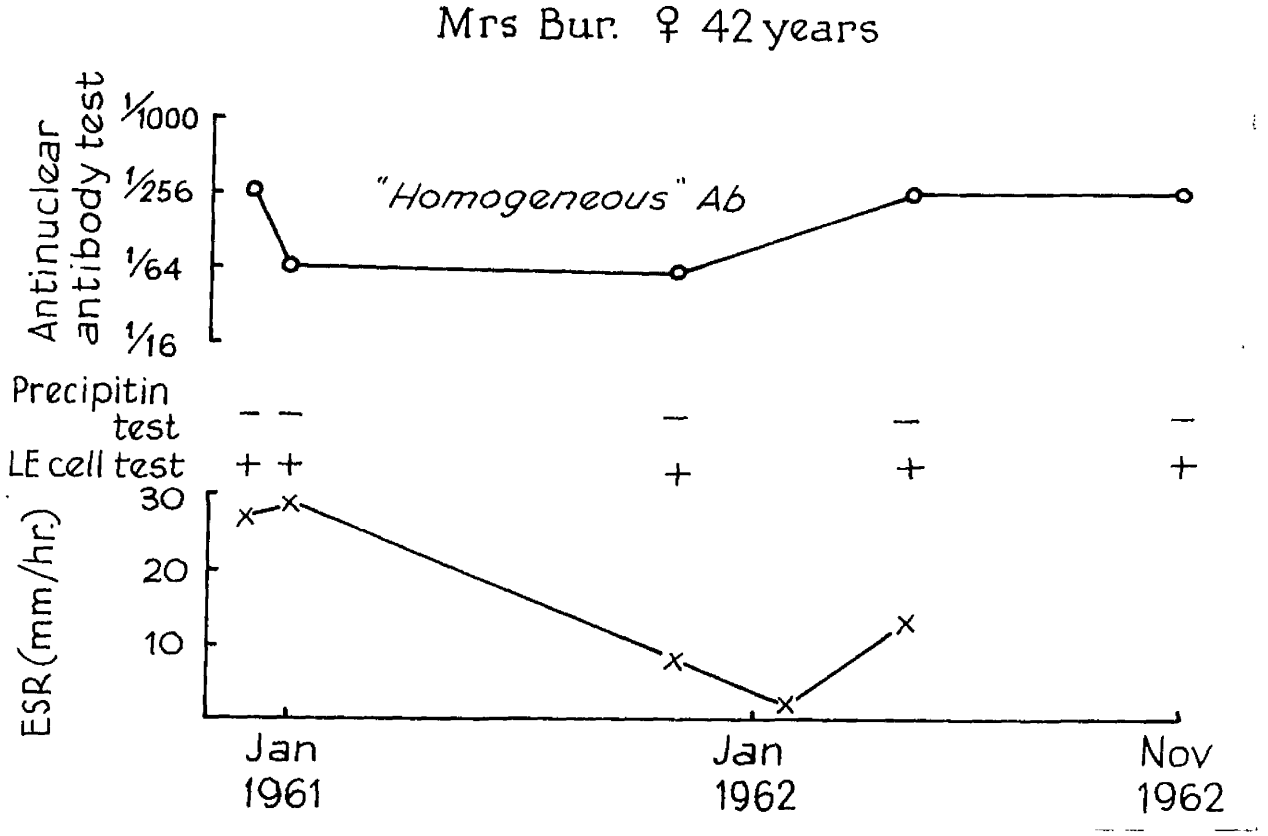


Fig. 8/6 Serial studies on systemic lupus erythematosus patient (Mrs Bur.)

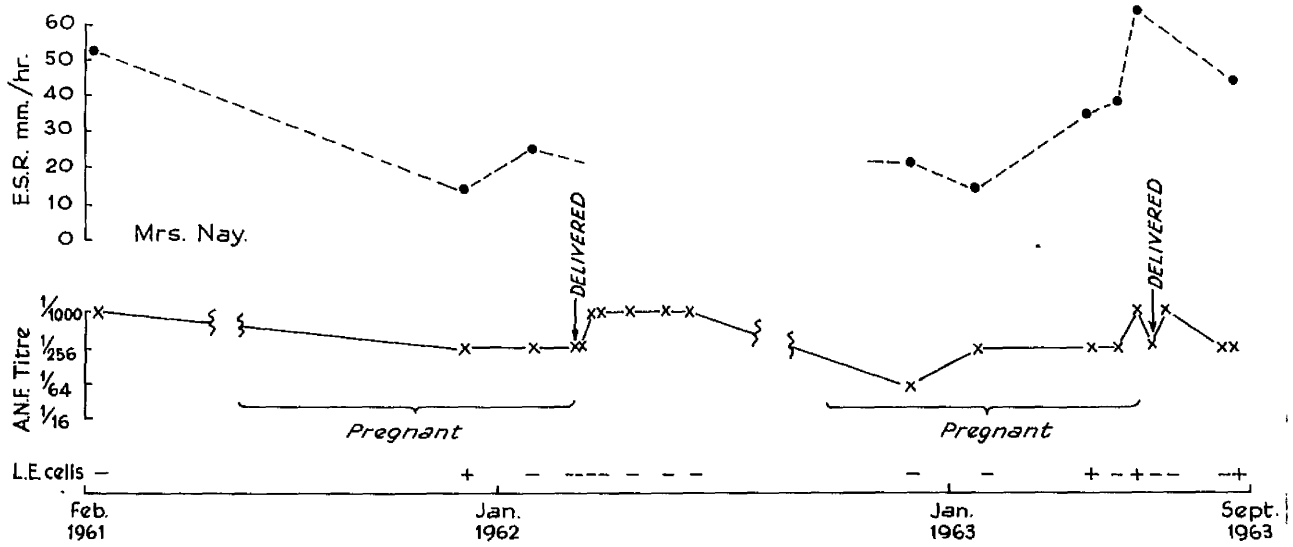


Fig. 8/7 Serial studies on systemic lupus erythematosus patient (Mrs Nay.)

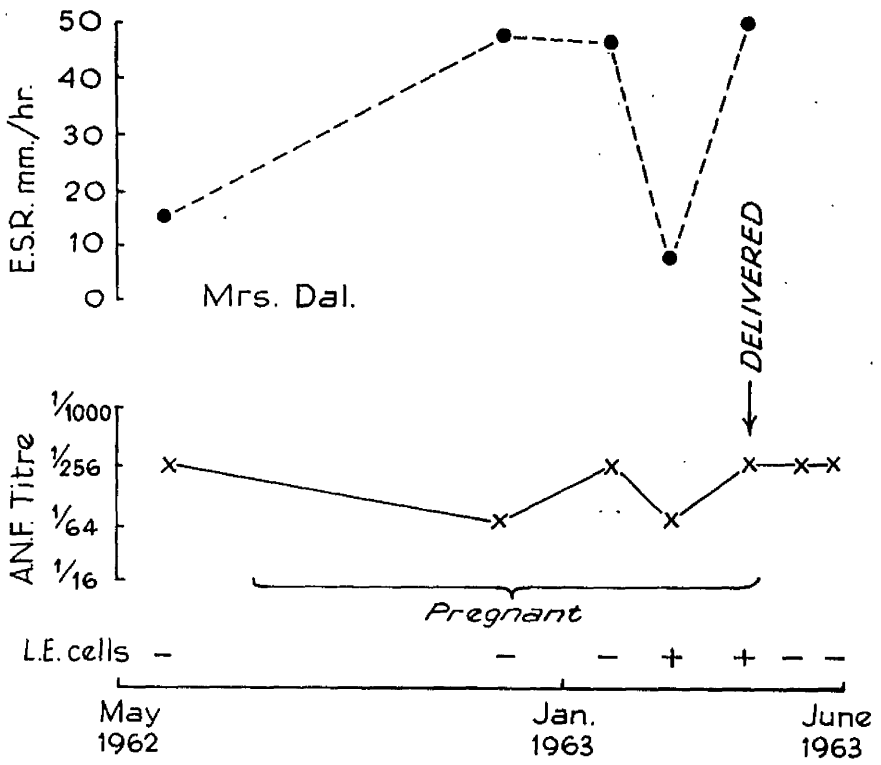


Fig. 8/8 Serial studies on systemic lupus erythematosus patient (Mrs Dal.)

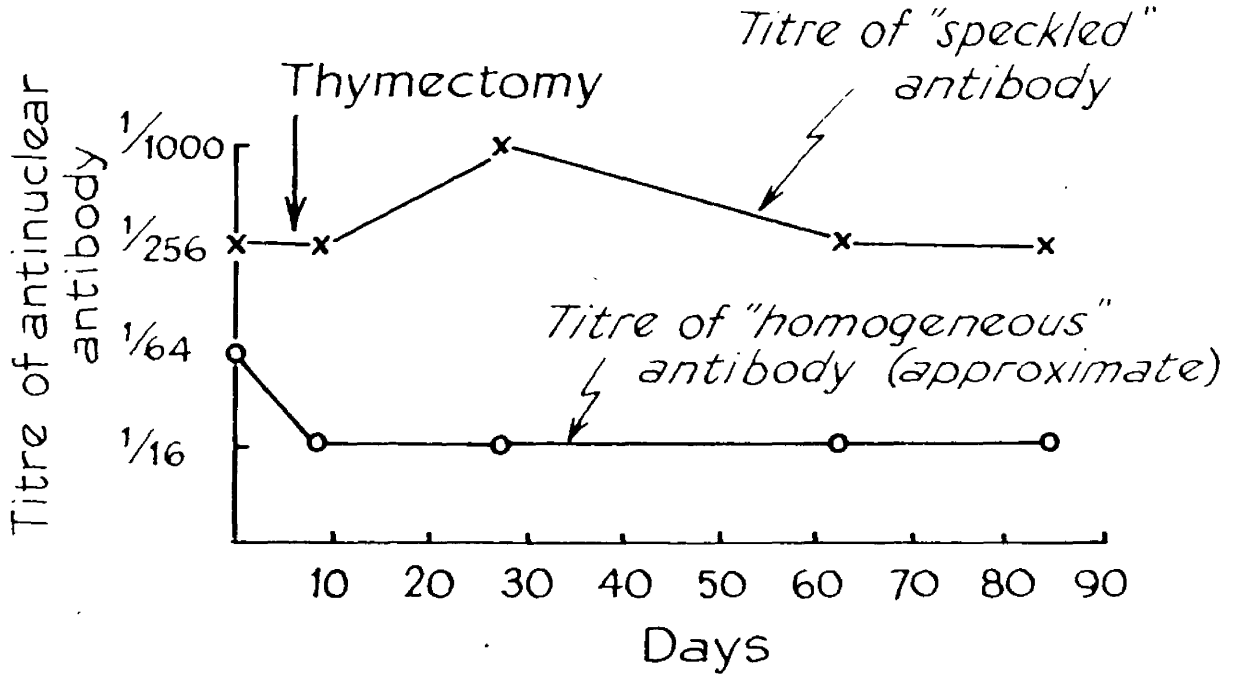


Fig. 8/9 Effect of thymectomy on antinuclear antibody titre in systemic lupus erythematosus patient (Mrs Bro.)

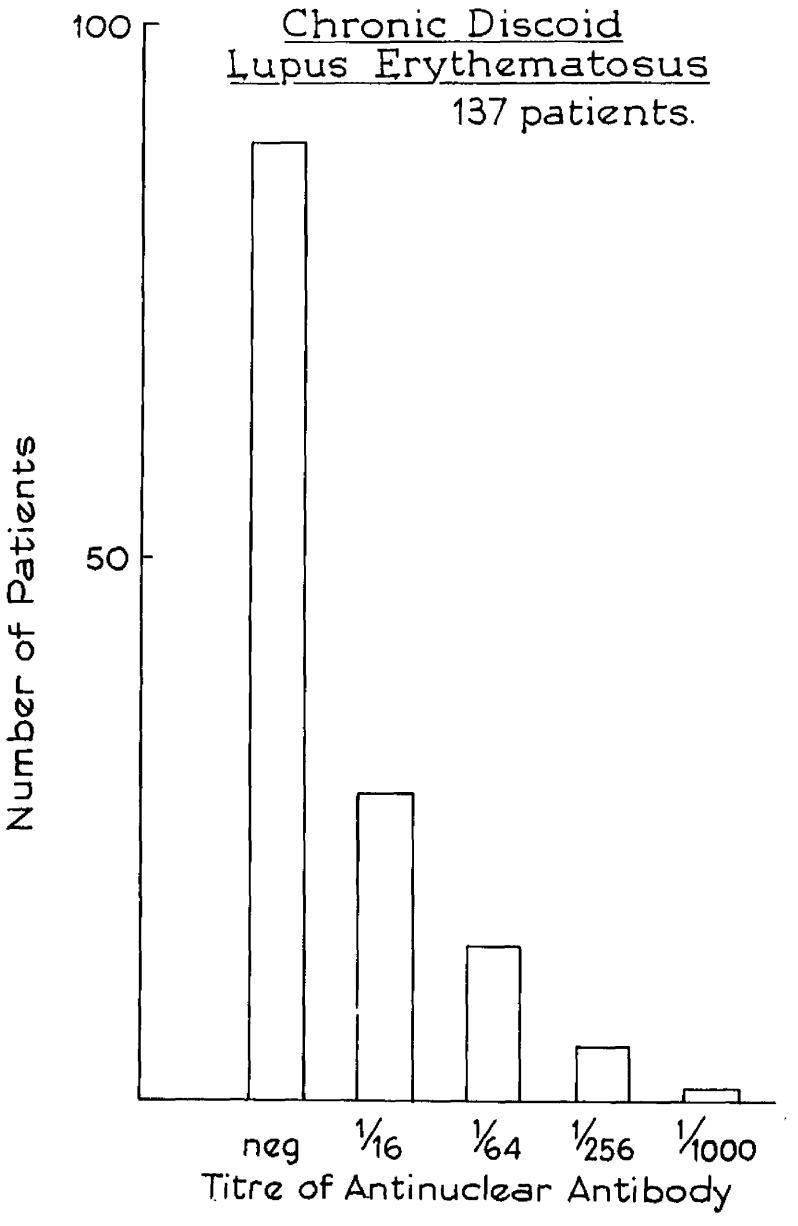


Fig. 8/10 Titres of antinuclear antibodies (all types) in chronic discoid lupus erythematosus.

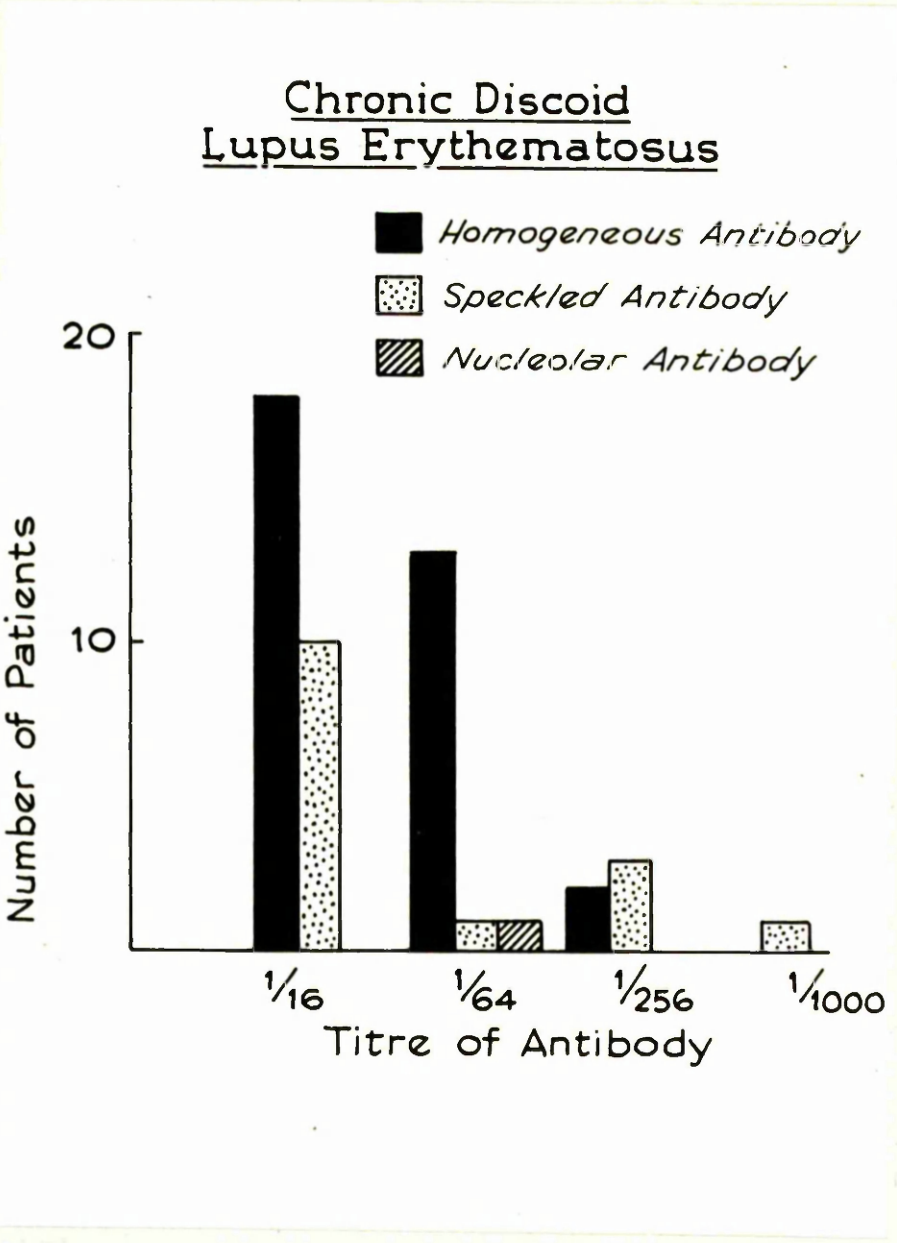


Fig. 8/11 Titres of different systems of antinuclear antibodies in chronic discoid lupus erythematosus.

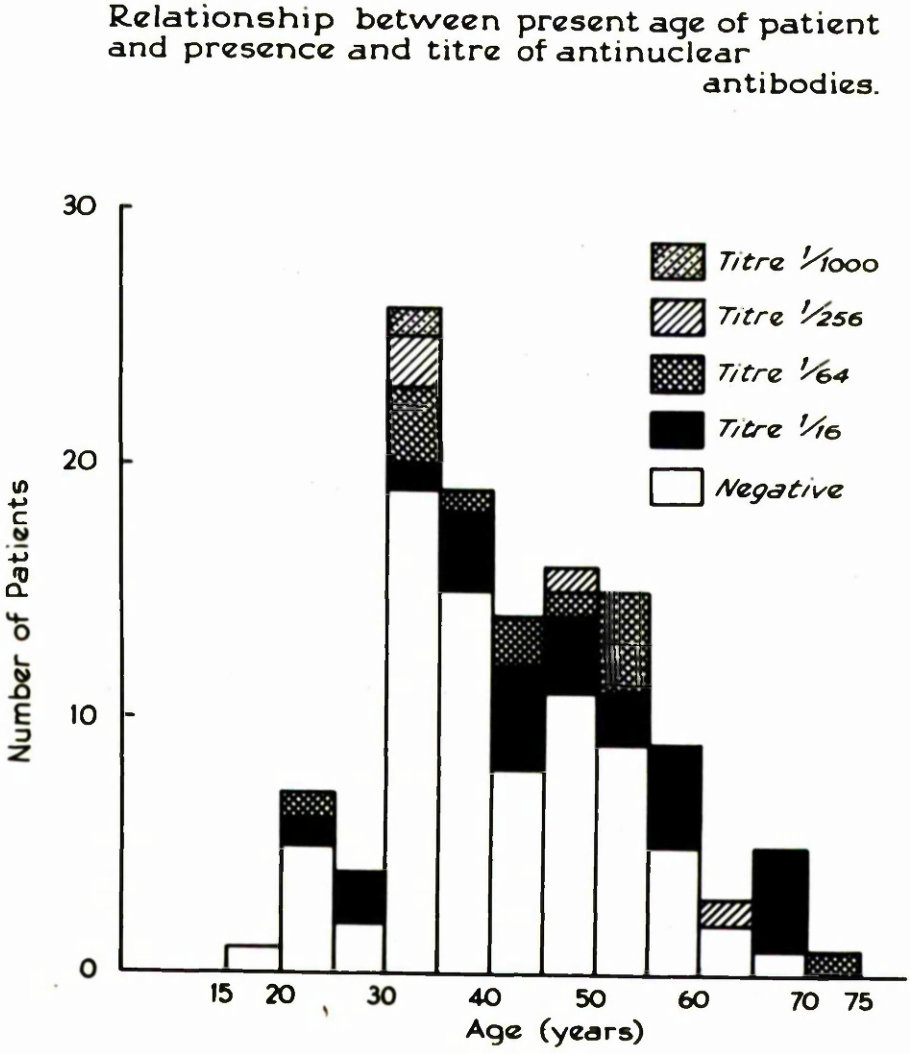


Fig. 8/12 Relationship between present age of patient and presence and titre of antinuclear antibodies in chronic discoid lupus erythematosus.

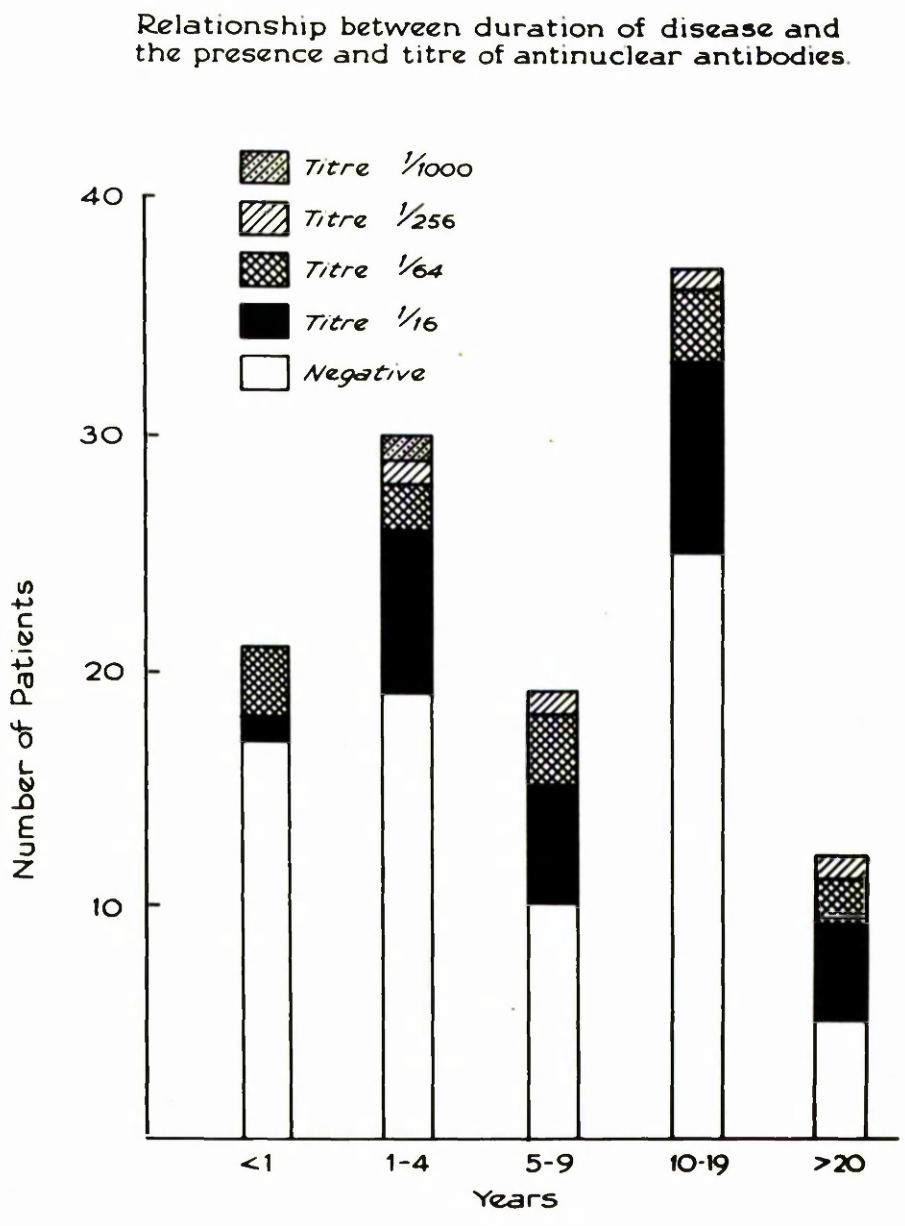


Fig. 8/13 Relationship between duration of disease and presence and titre of antinuclear antibodies in chronic discoid lupus erythematosus.

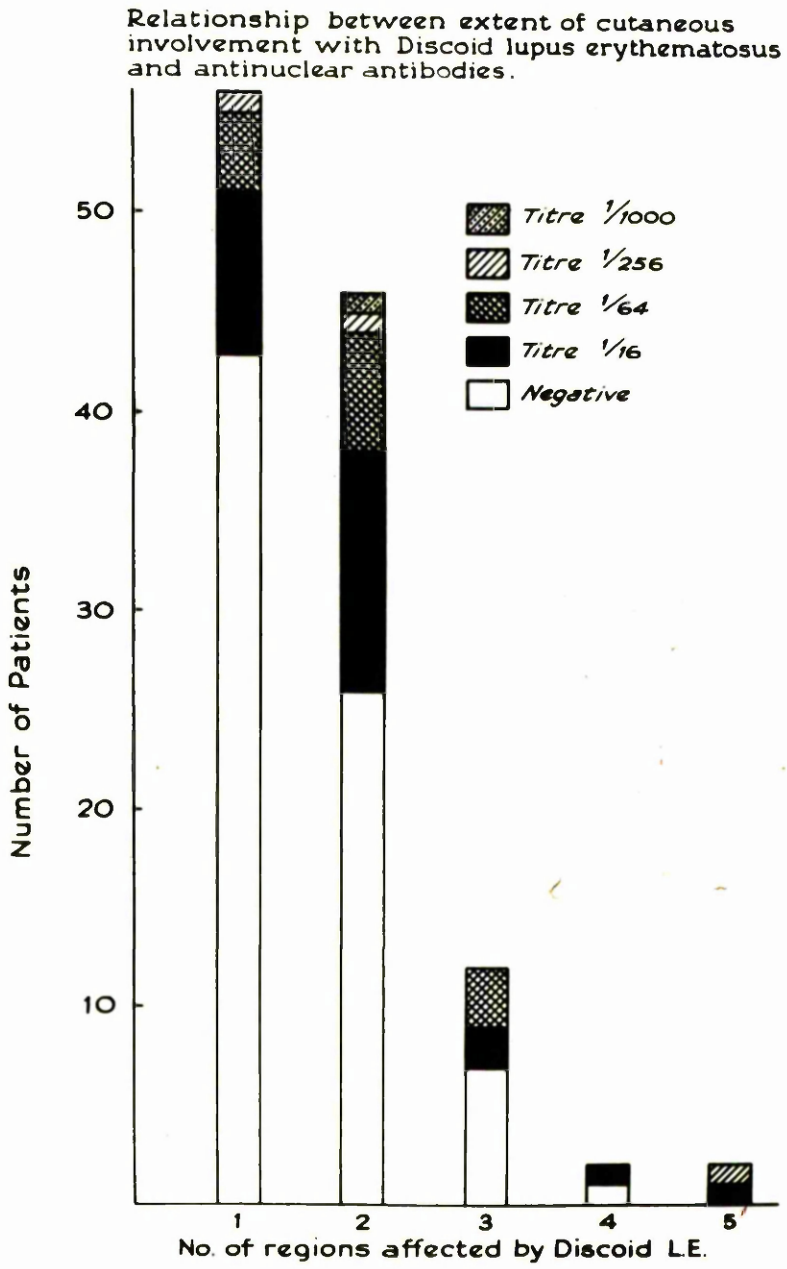


Fig. 8/14 Relationship between extent of cutaneous involvement with chronic discoid lupus erythematosus and antinuclear antibodies.

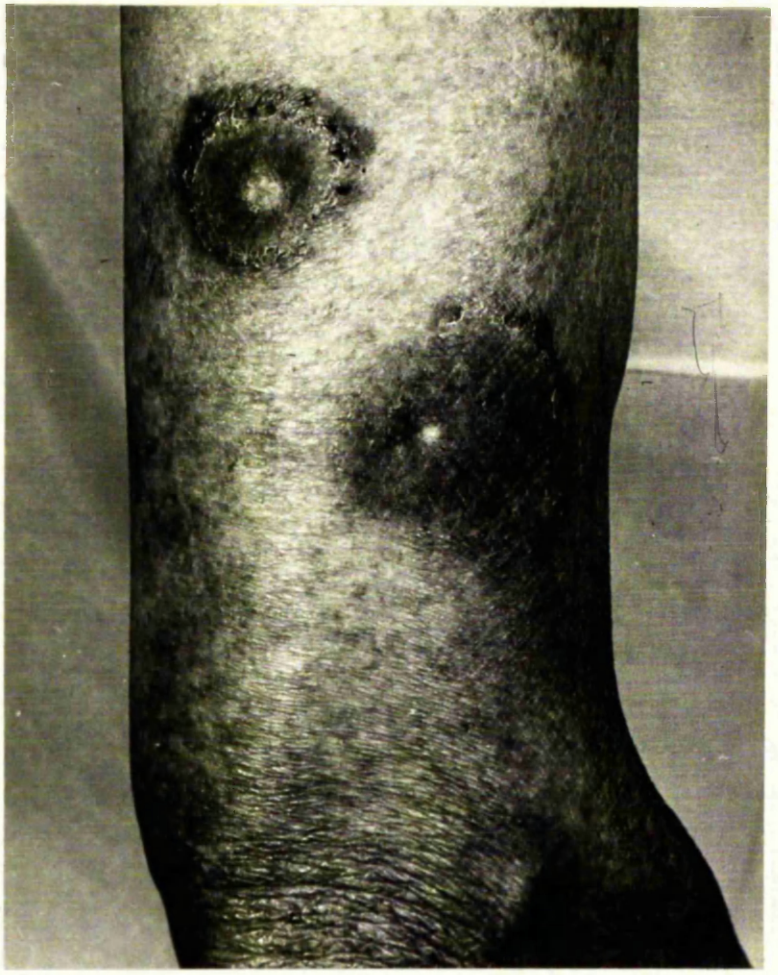


Fig. 8/15 Annular lesions in discoid lupus erythematosus.

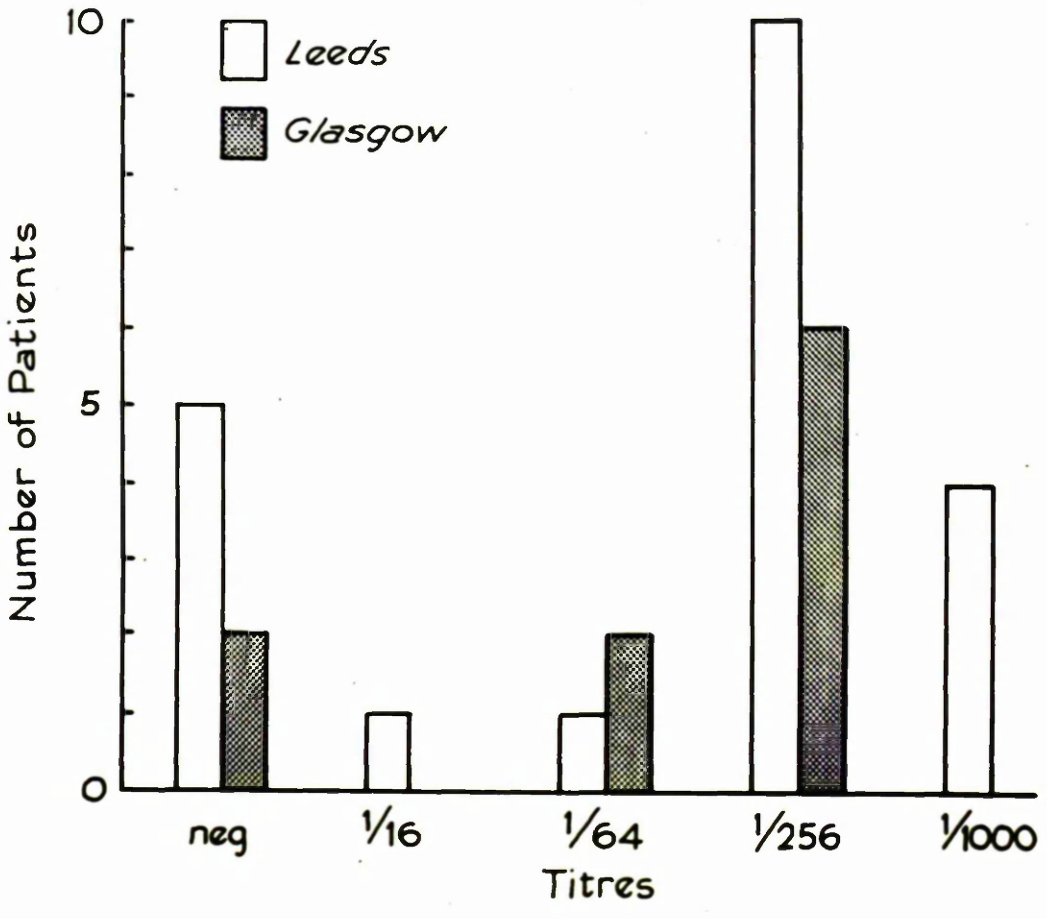


Fig. 8/16 Titres of antimuclear antibodies (all types) in Glasgow and Leeds progressive systemic sclerosis patients.

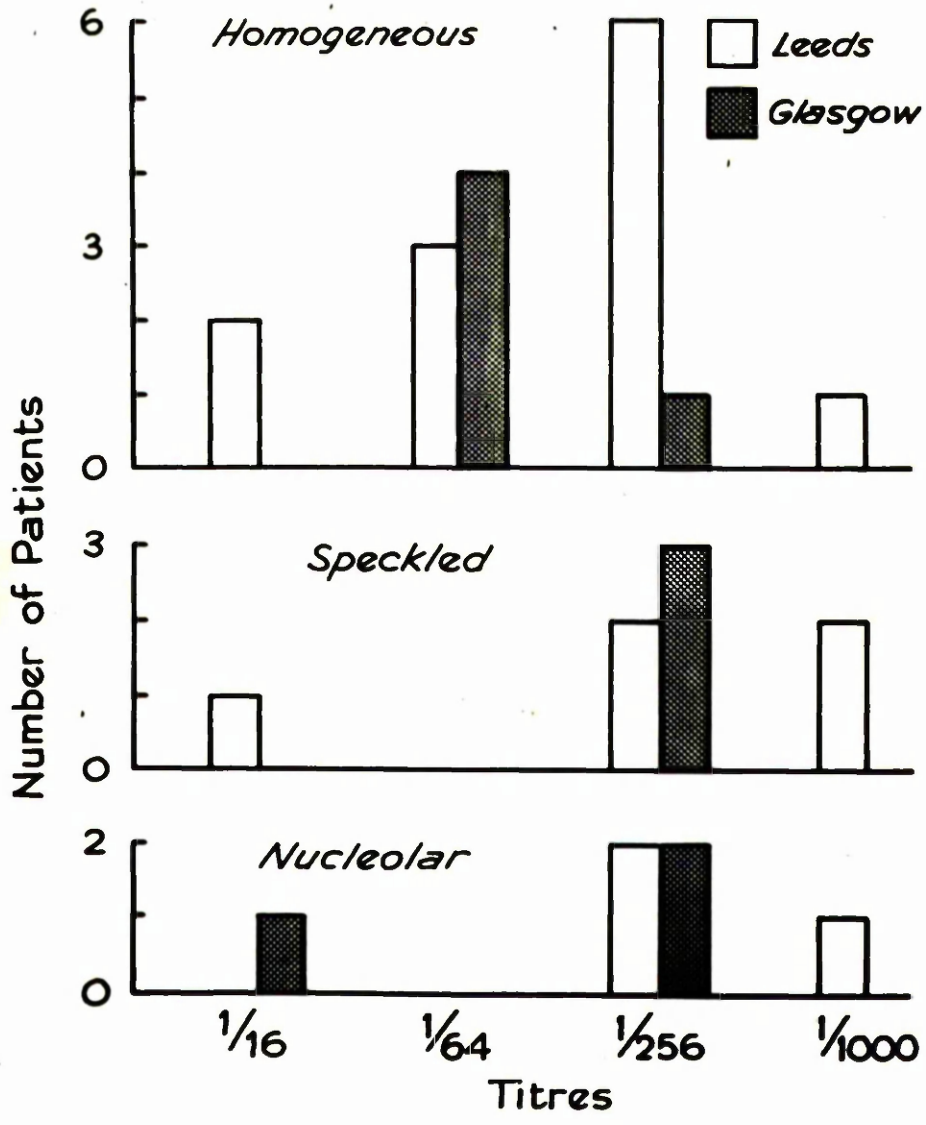


Fig. 8/17 Titres of different systems of antinuclear antibodies in Glasgow and Leeds progressive systemic sclerosis patients.

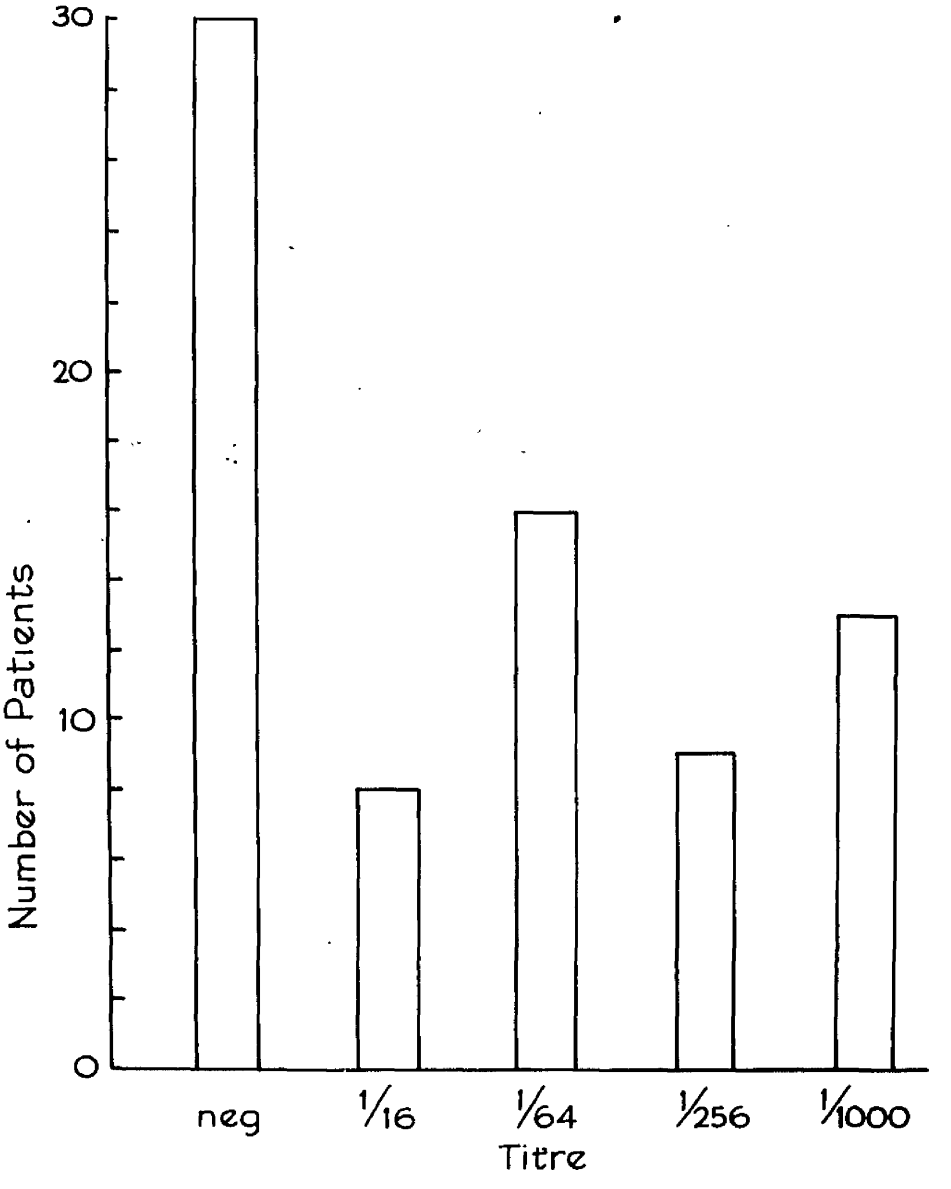


Fig. 8/18 Titres of antinuclear antibodies (all types) in Sjögren's syndrome.

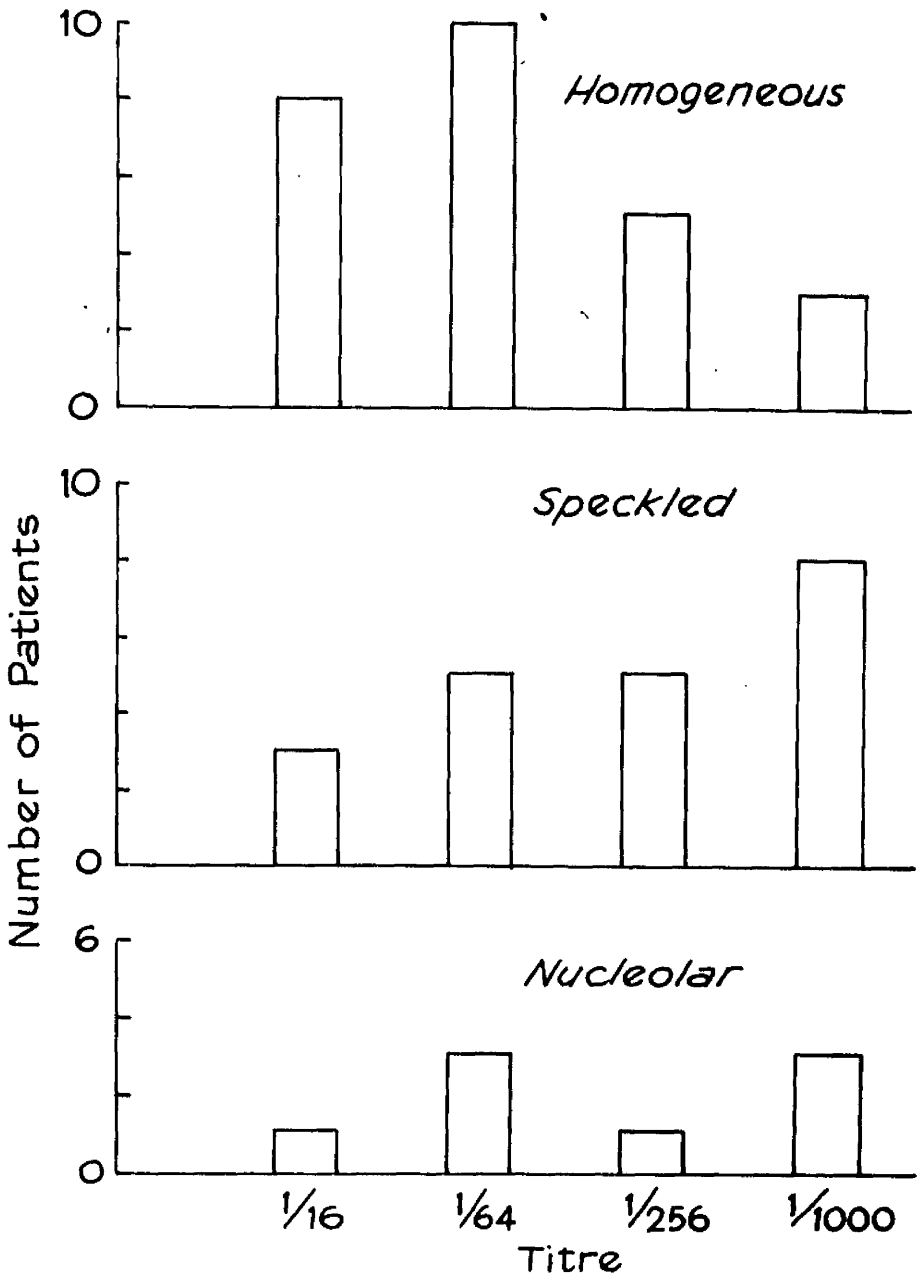


Fig. 8/19 Titres of different systems of antinuclear antibodies in Sjögren's syndrome.

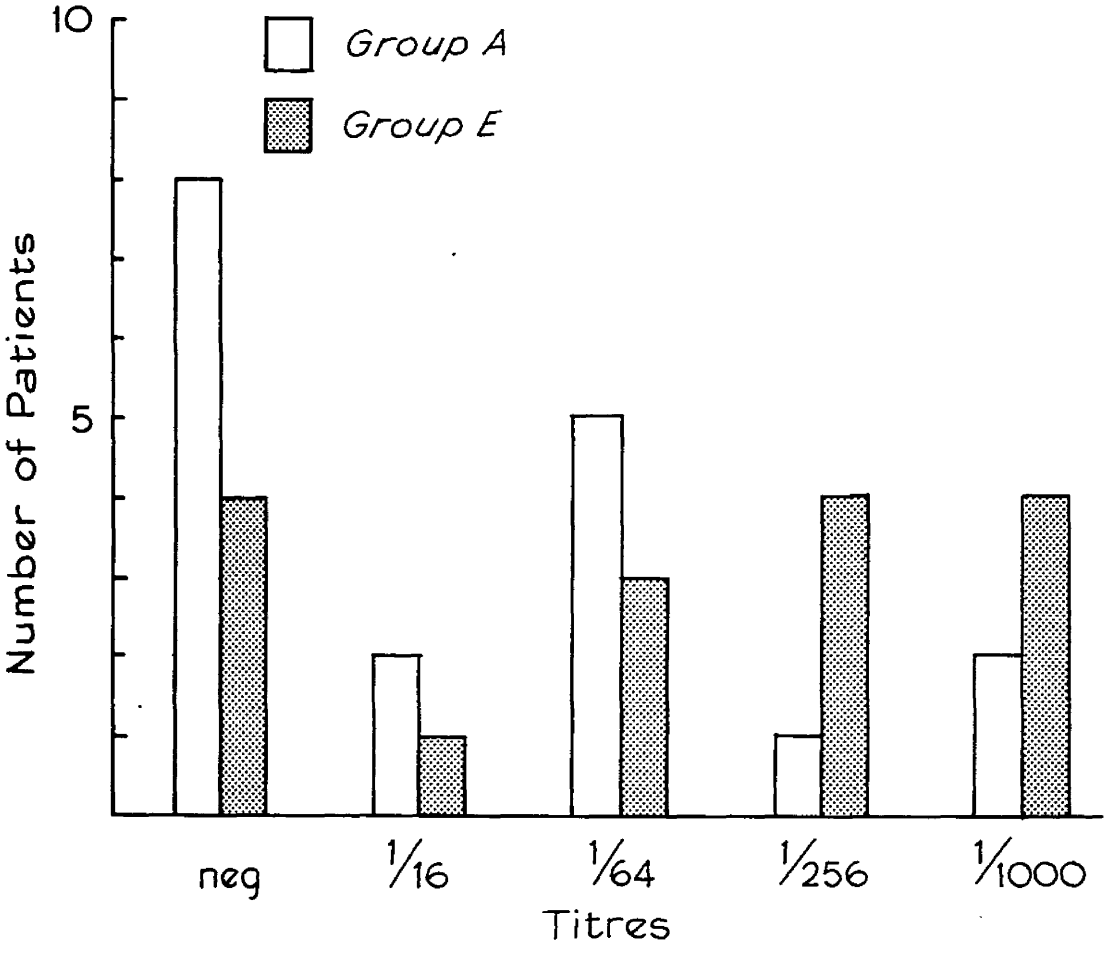


Fig. 8/20 Titres of antinuclear antibodies (all types) in groups A & E Sjogren's syndrome patients.

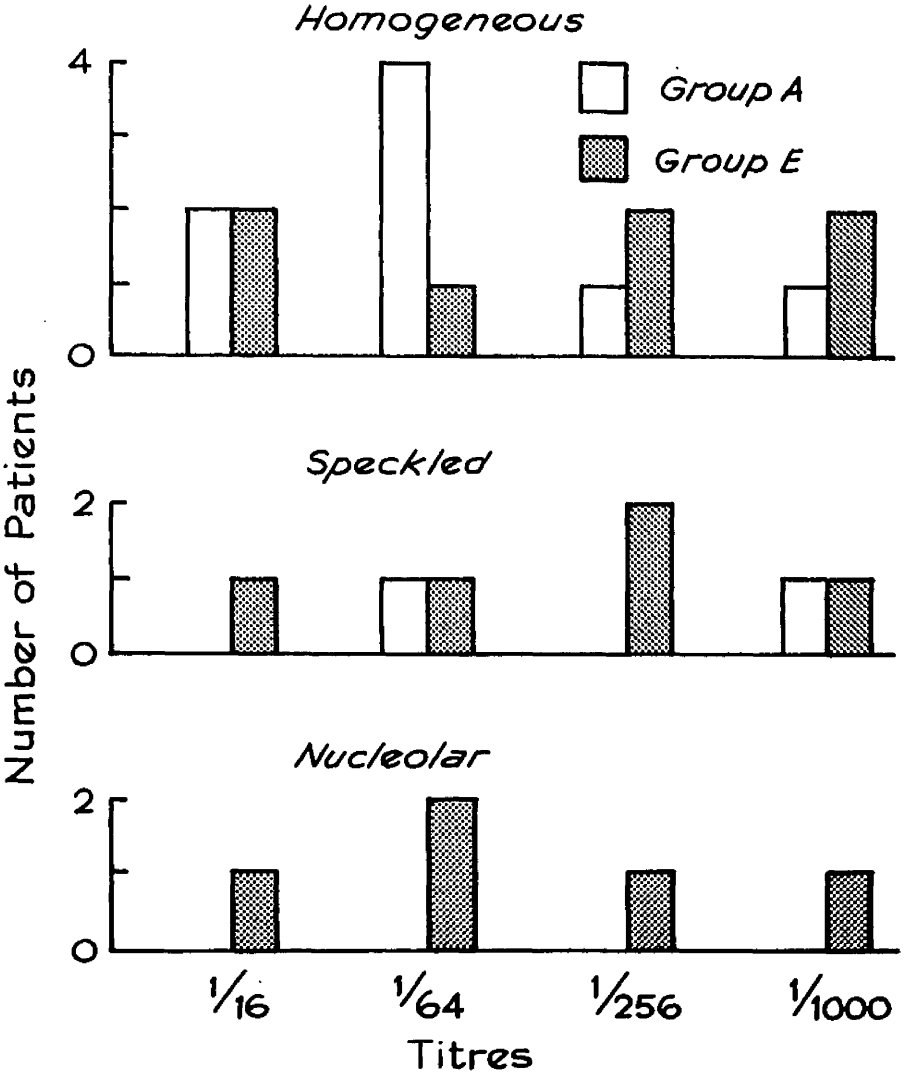


Fig. 3/21 Titres of different systems of antinuclear antibodies in groups A & E Sjögren's syndrome patients.

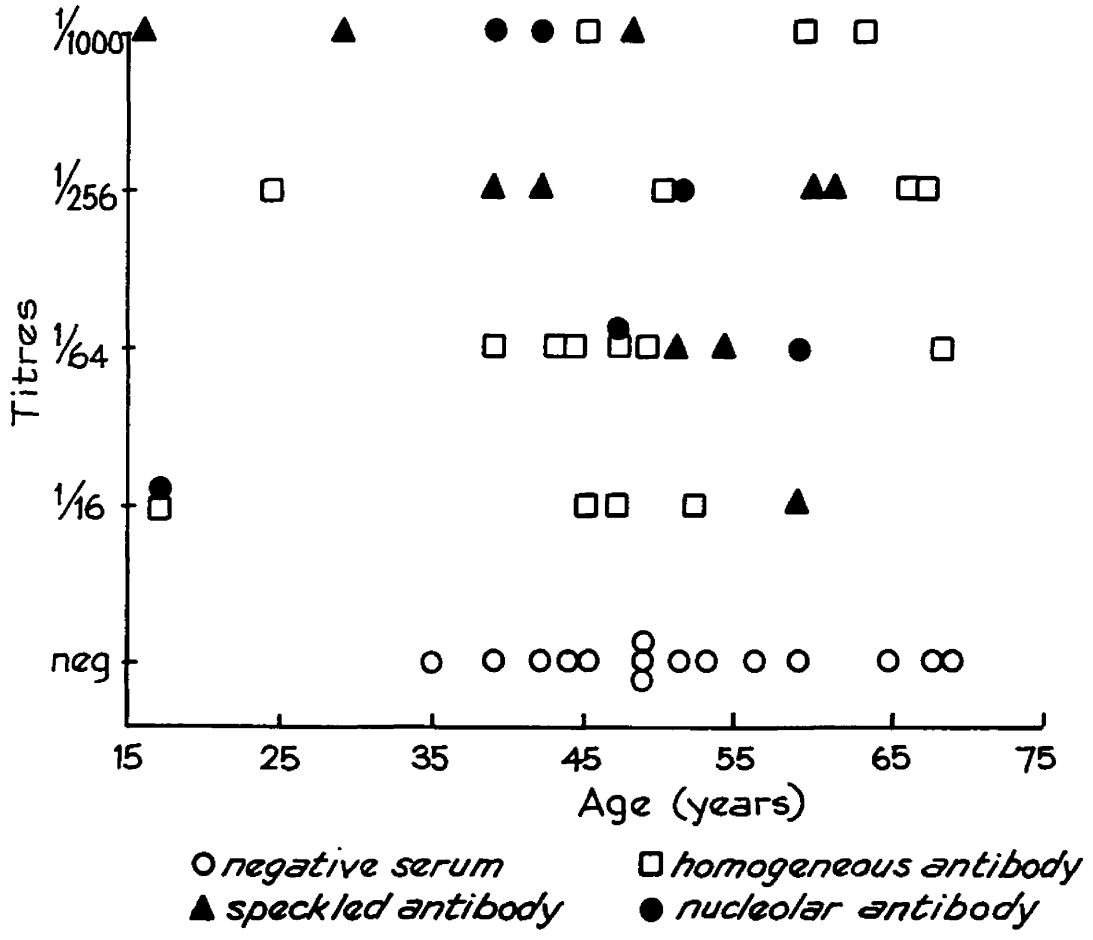


Fig. 8/22 Relationship between present age of patient and titre of antinuclear antibodies in Sjögren's syndrome.

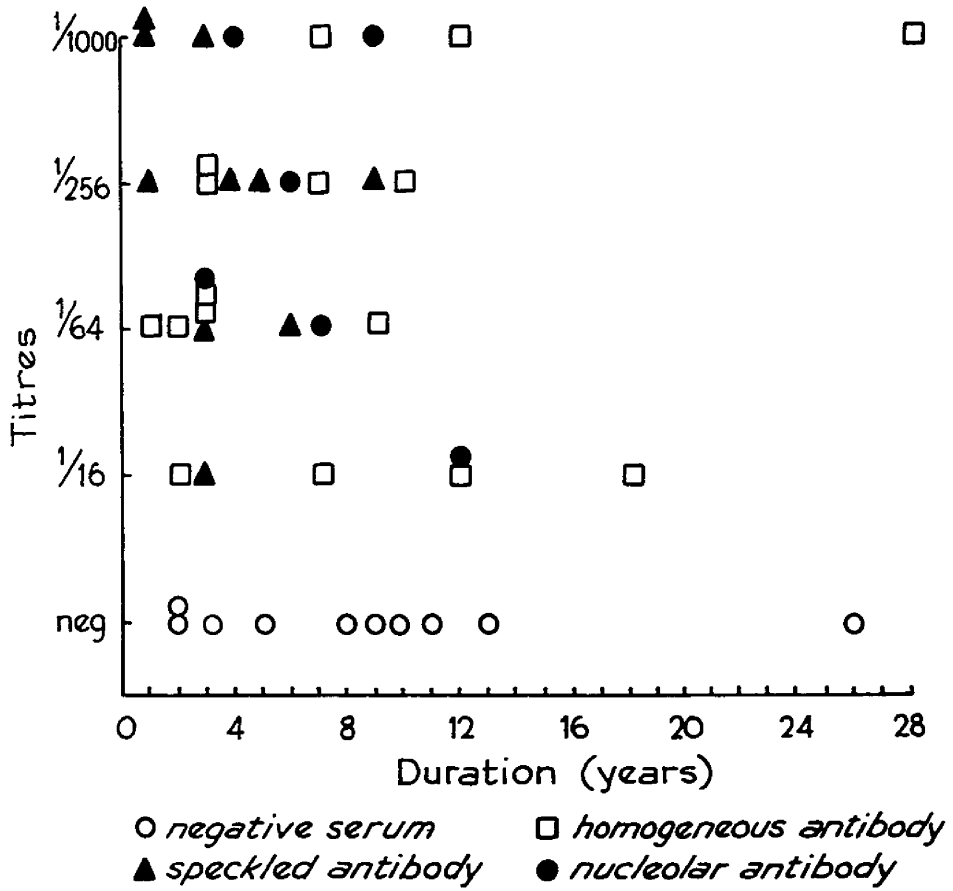


Fig. 8/23 Relationship between duration of disease and titre of antinuclear antibodies in Sjögren's syndrome.

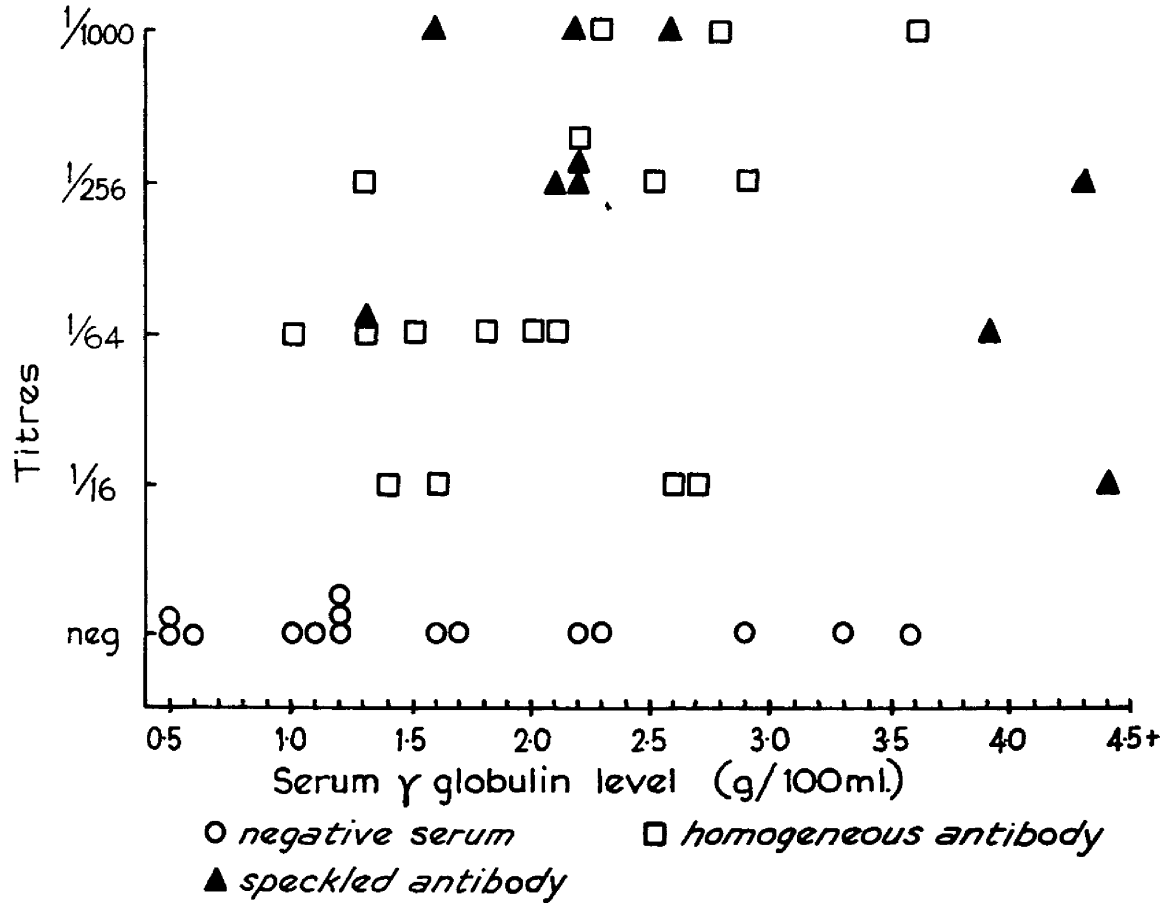


Fig. 3/24 Relationship between serum γ globulin level and antinuclear antibodies in Sjogren's syndrome.

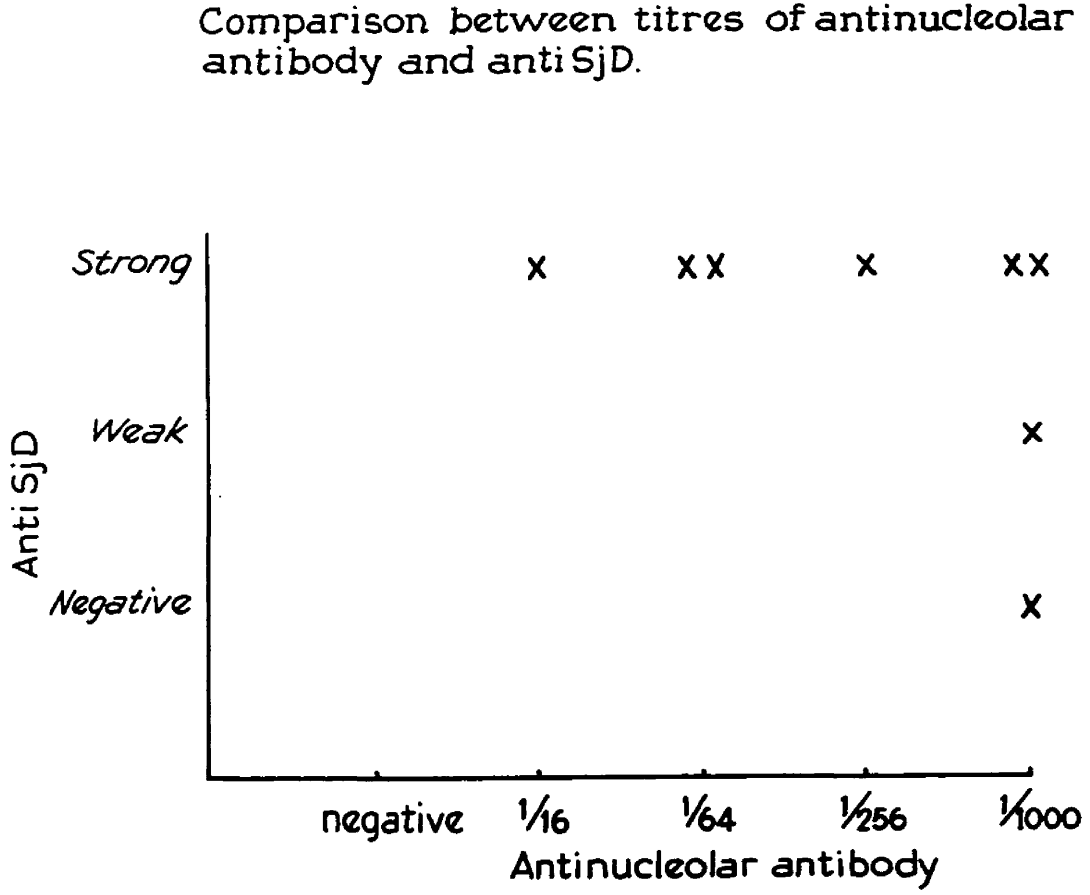


Fig. 8/25 Comparison between antinucleolar antibody and anti-SjD precipitin in Sjögren's syndrome.

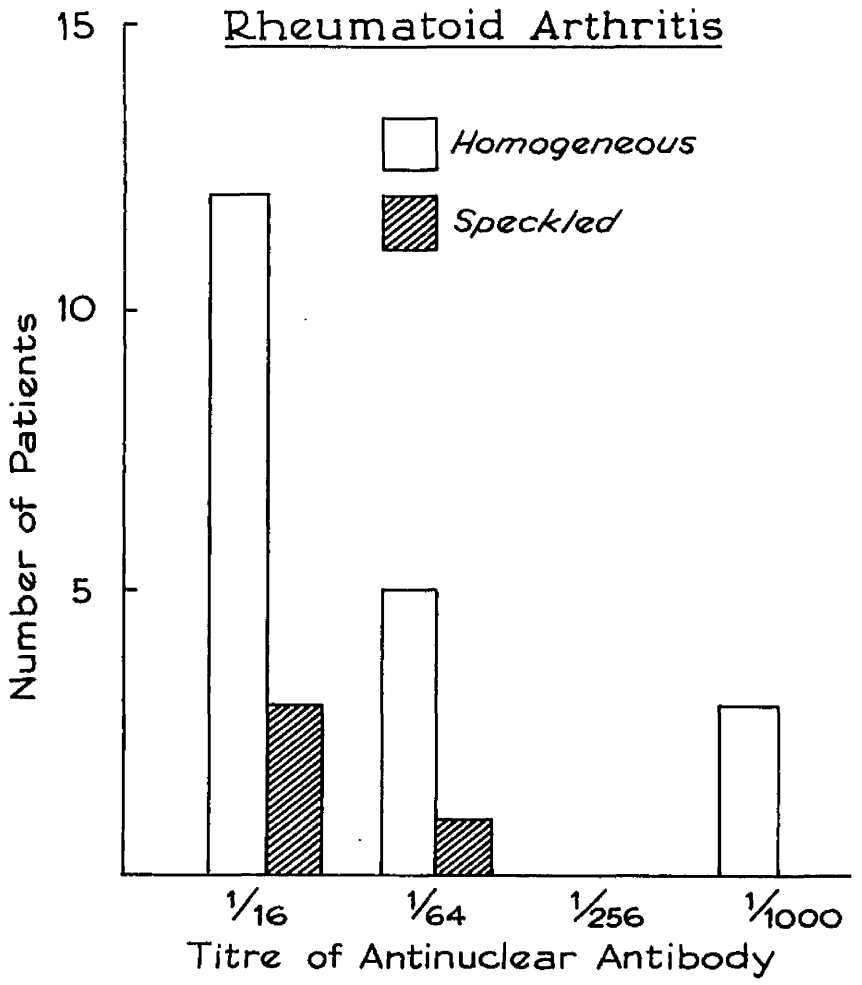


Fig. 8/26 Titres of antinuclear antibodies (all types) in rheumatoid arthritis.

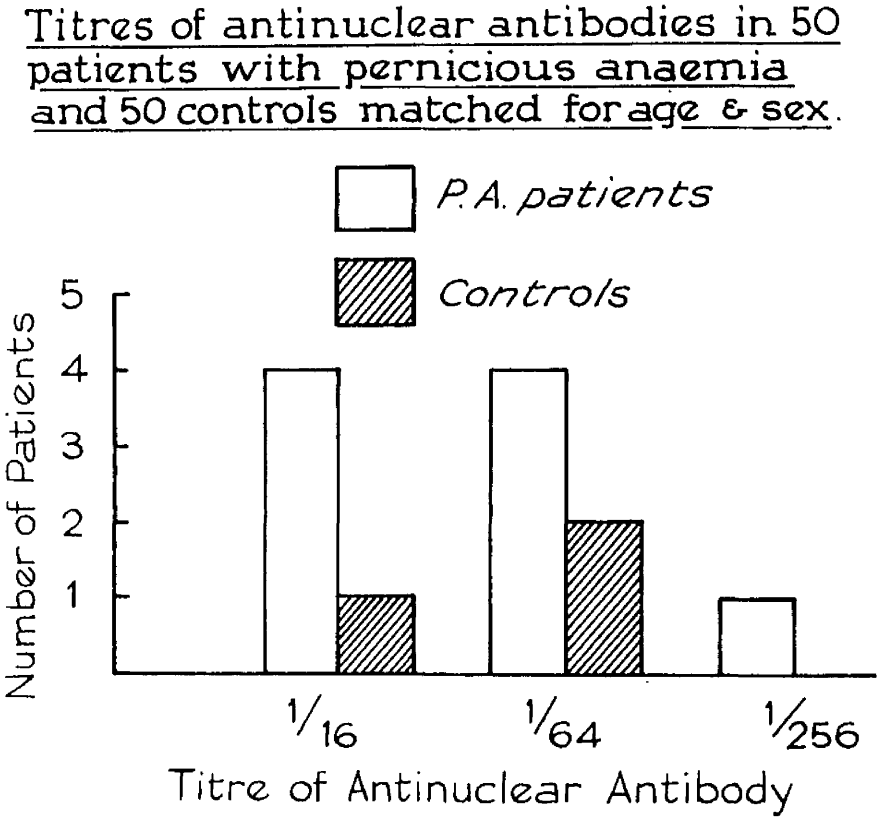


Fig. 8/27 Titres of antinuclear antibodies (all types) in pernicious anaemia.

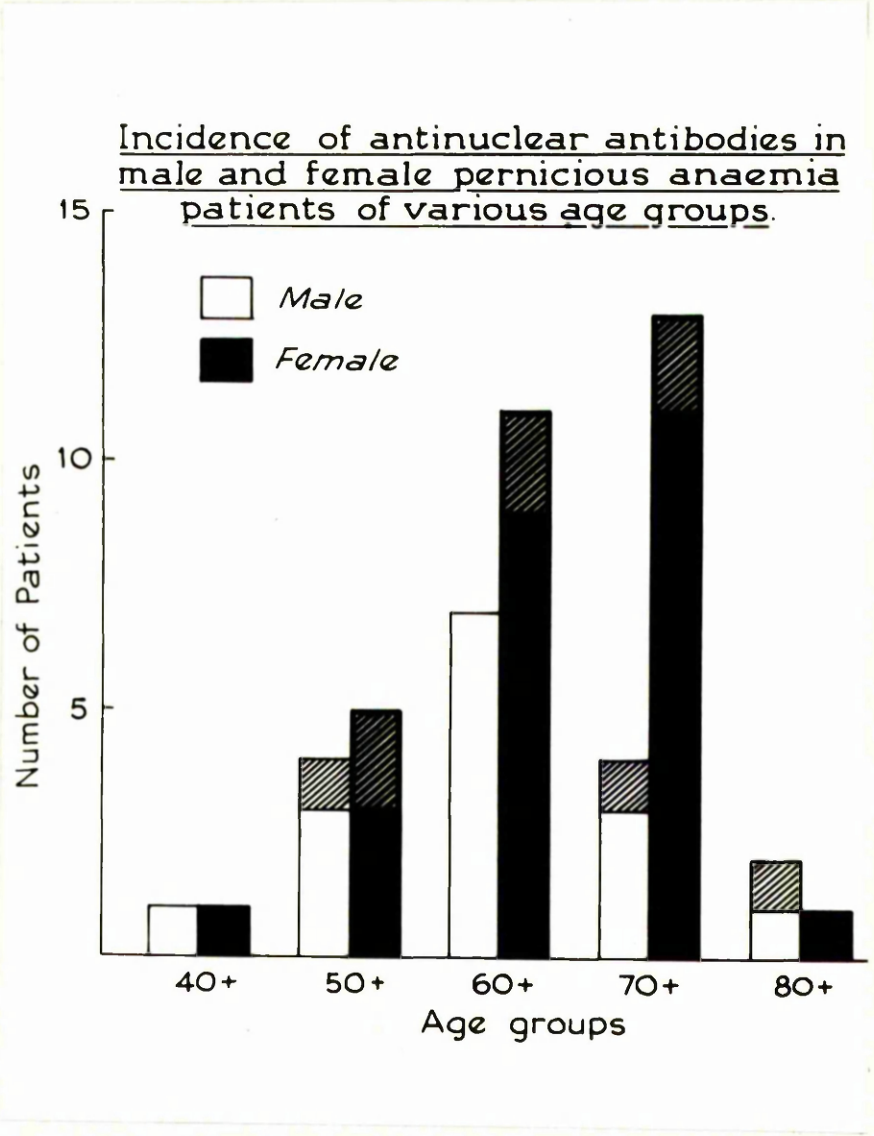


Fig. 8/28 Incidence of antimuclear antibodies in male and female pernicious anaemia patients of various age groups.

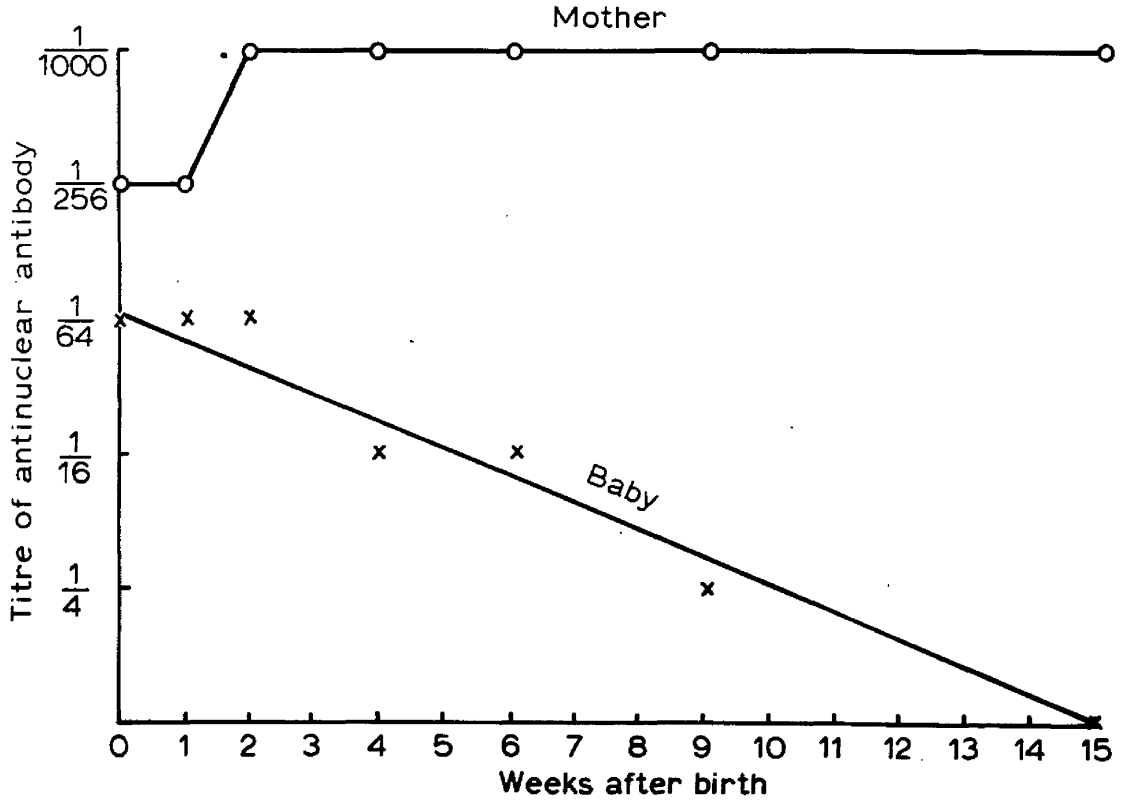


Fig. 9/1 Transplacental passage of antinuclear antibody. Serial studies on mother (Mrs Nay.) and her baby after first pregnancy. The half life of the antibody in the baby was 17.5 days.

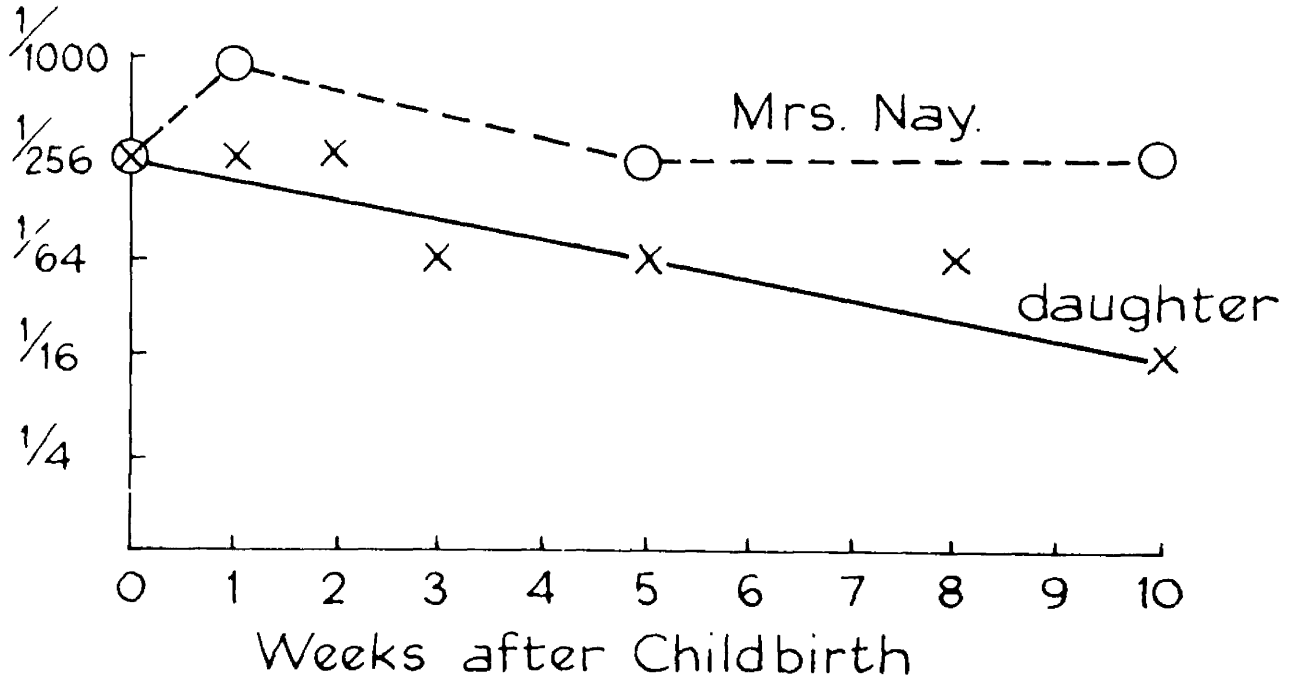


Fig. 9/2 Transplacental passage of antinuclear antibody. Serial studies on mother (Mrs Nay.) and her baby after second pregnancy. The half life of the antibody in the baby was 16.5 days.

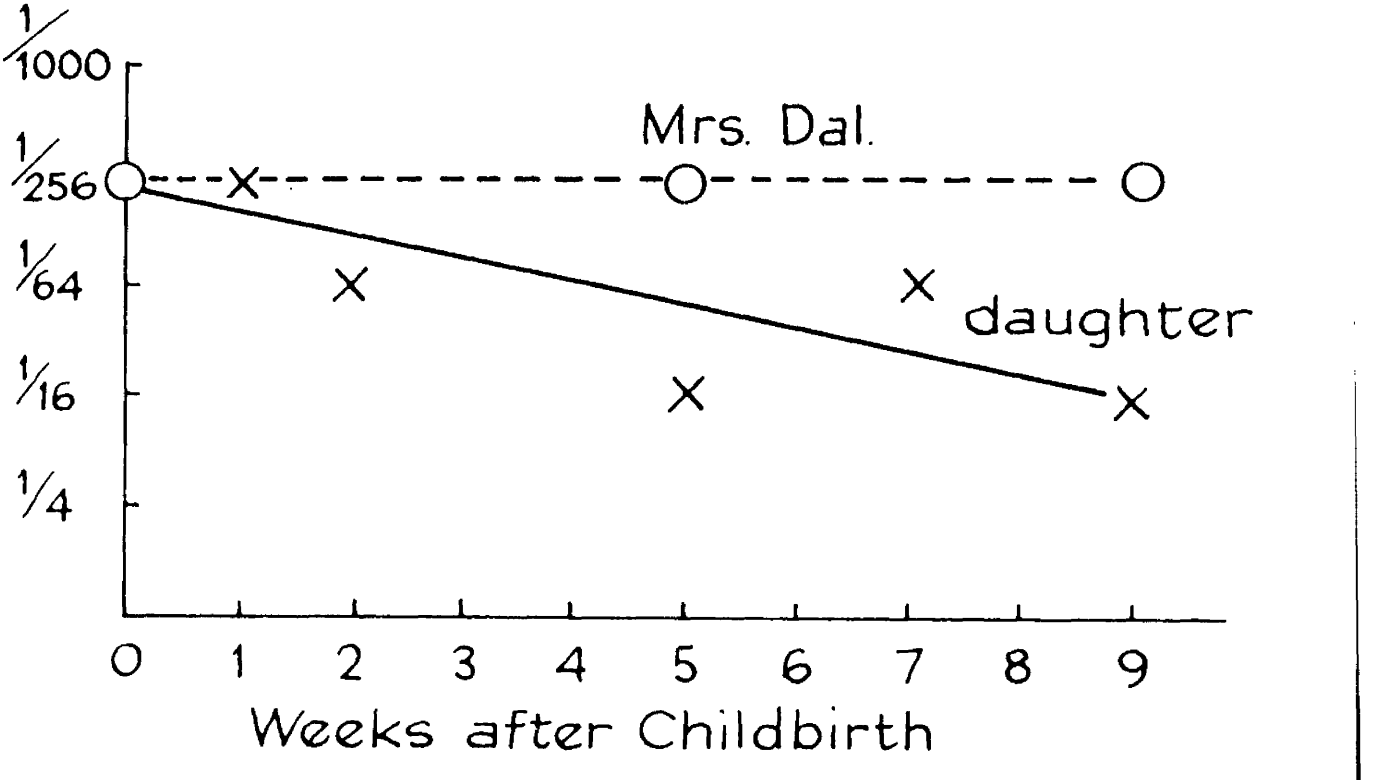


Fig. 9/3 Transplacental passage of antinuclear antibody. Serial studies on mother (Mrs Dal.) and her baby showing that the half life of the antibody in the baby was 16 days.

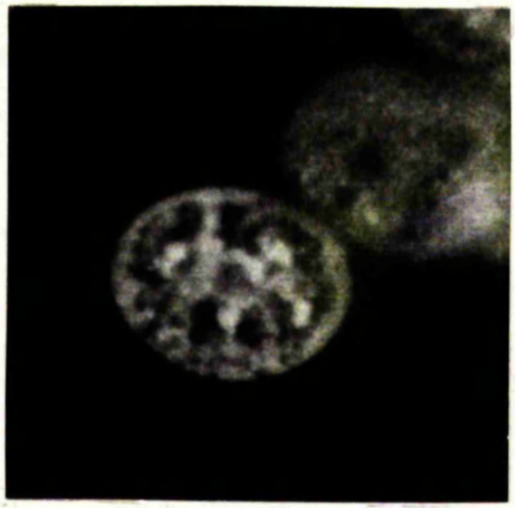


Fig. 10/1 Early prophase stained with "homogeneous" antinuclear serum (Dun.). Coverslip culture of HeLa cells x1500.

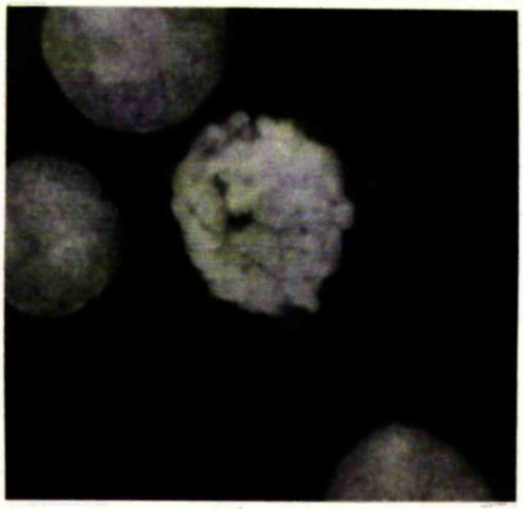


Fig. 10/2 Late prophase stained with "homogeneous" antinuclear serum (War.) x 1500.

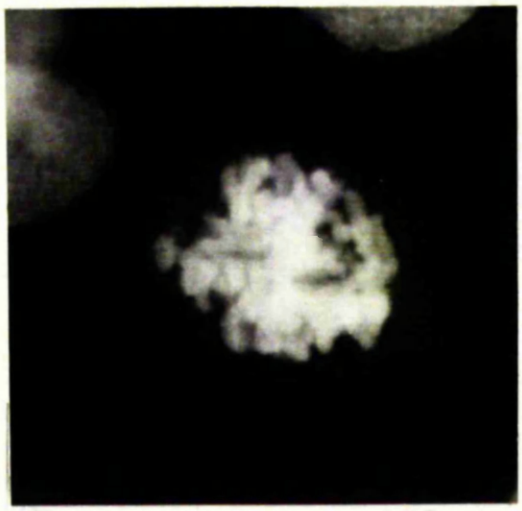


Fig. 10/3 Late prophase stained with "homogeneous" antinuclear serum (War.) x1500.

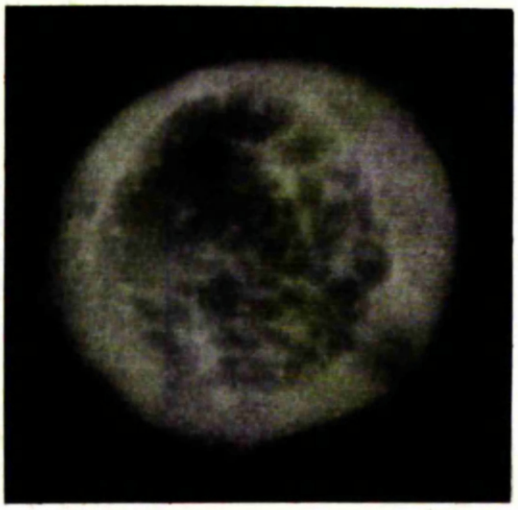


Fig. 10/4 Early prophase stained with "speckled" antinuclear serum (Tra.). The "speckled" antigen is present in the cytoplasm and the chromosomes are unstained x1500.

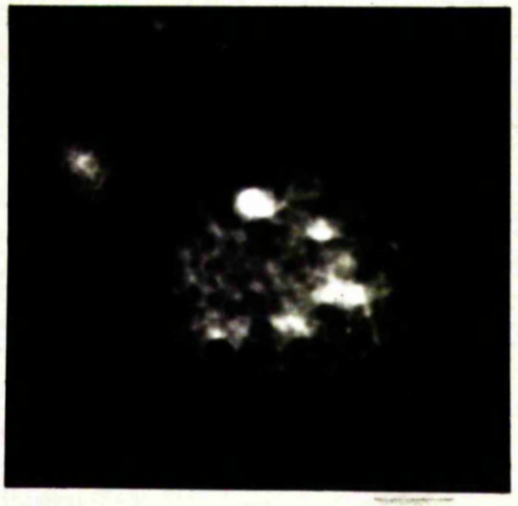


Fig. 10/5 Early prophase stained with antinucleolar serum (Mui.) Discrete nucleoli are stained although chromosome condensation is well advanced x1500.

Fig. 10/6 Late prophase stained with antinucleolar serum (Scr.) The nucleolar antigen is present in the cytoplasm and the chromosomes are unstained x1500.

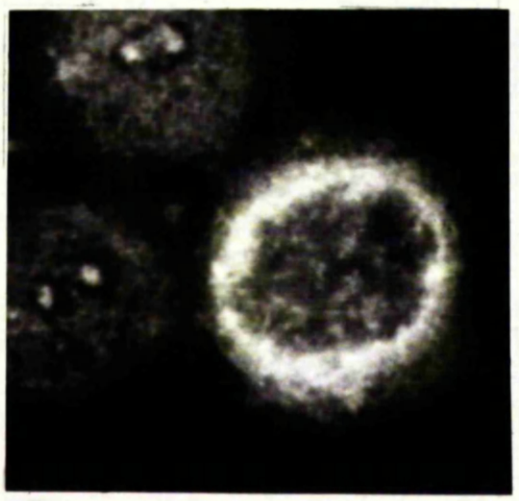


Fig. 10/7 Late prophase stained with antinucleolar serum (Scr.) The nucleolar antigen is absent from the chromosomes but present in the nucleoli of adjacent interphase nuclei x1500.

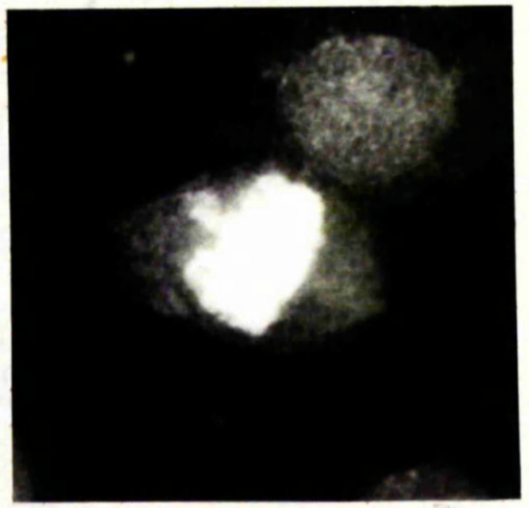


Fig. 10/8 Metaphase stained with "homogeneous" antinuclear serum (War.) The chromosomal plate is stained brightly x1500.

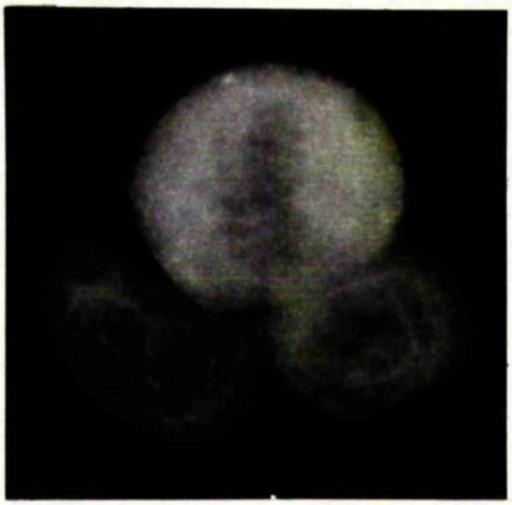


Fig. 10/9 Metaphase and early telophase stained with "speckled" antinuclear serum (Tra.) The chromosomes are unstained x1500.

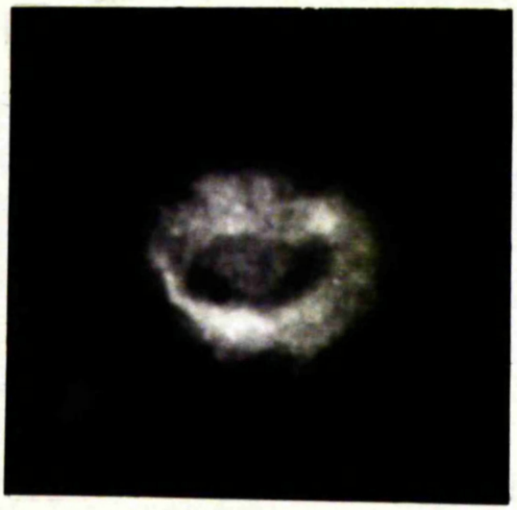


Fig. 10/10 Metaphase stained with antinucleolar serum (Scr.) The antigen is restricted to the cytoplasm. The chromosomal plate is unstained but shows a central ill-defined area of autofluorescence x1500.

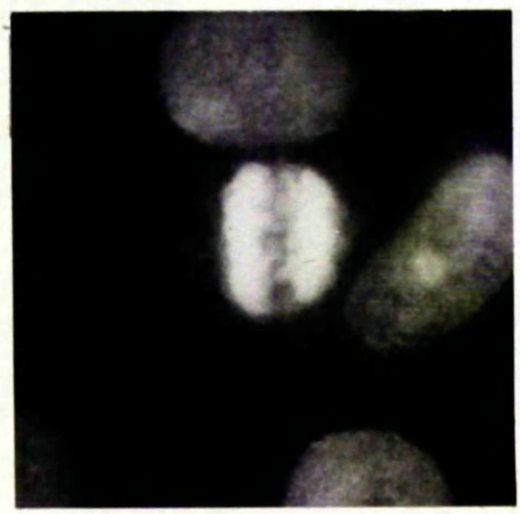


Fig. 10/11 Early anaphase stained with "homogeneous" antinuclear serum (War.) showing bright specific staining of chromosomal plates x1500.

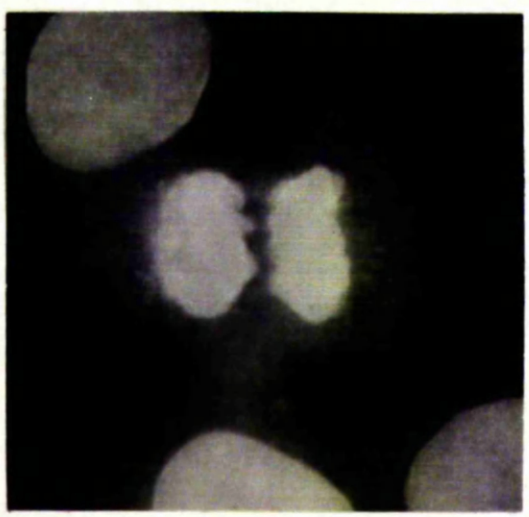


Fig. 10/12 Late anaphase stained with "homogeneous" antinuclear serum (War.) x1500.

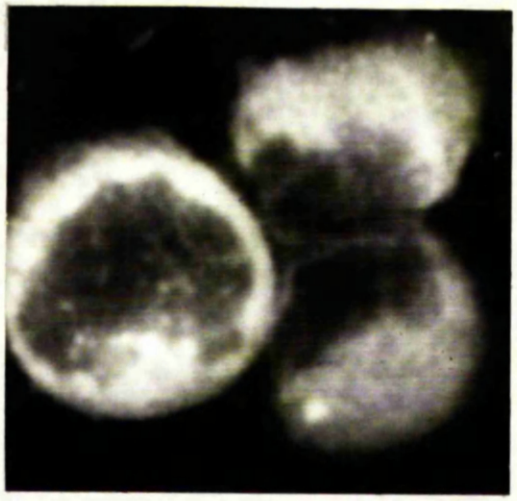


Fig. 10/13 Anaphase stained by "speckled" antinuclear serum (Tra.) showing that the antigen is in the cytoplasm and that the chromosomal plates are unstained. A cell in prophase is also included in the photograph x1500.

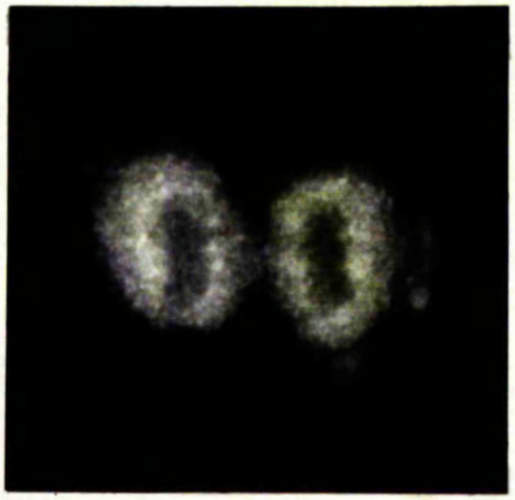


Fig. 10/14 Late anaphase stained with "speckled" antinuclear serum (Tra.) x1500.

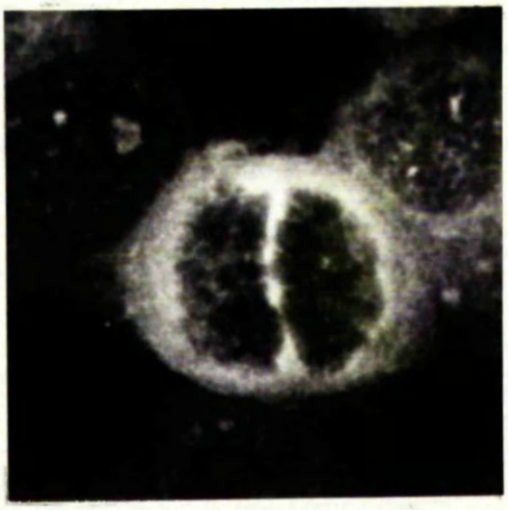


Fig. 10/15 Late anaphase stained with antinucleolar serum (Scr.) The chromosomes are unstained and the antigen is present mainly in the cytoplasm but several small nucleoli are forming in the daughter nuclei x1500.

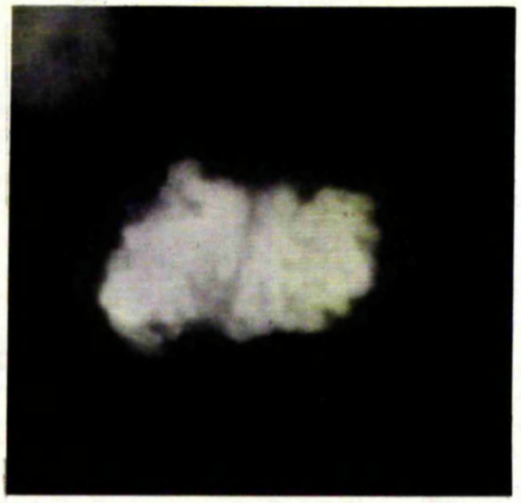


Fig. 10/16 Early telophase stained with "homogeneous" antinuclear serum (War.) The chromosomes now separated out from the plates are stained intensely x1500.

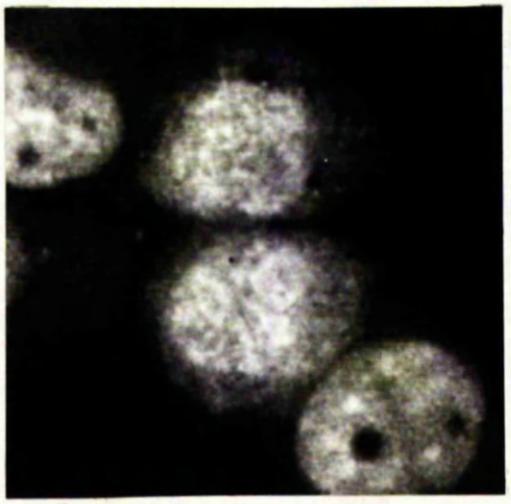


Fig. 10/17 Late telophase stained with "homogeneous" antinuclear serum (Dun.) The coarse chromatin pattern is brightly stained in the reforming daughter nuclei x1500.

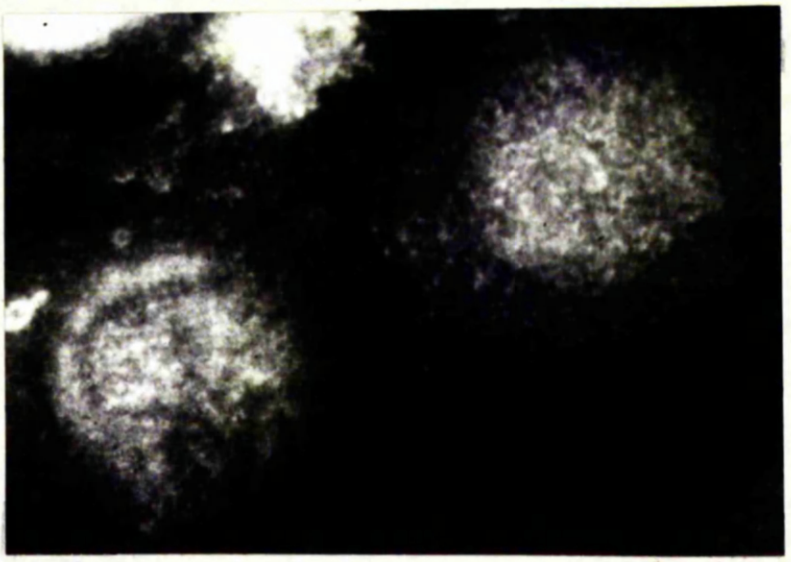


Fig. 10/18 Telophase stained with "speckled" antinuclear serum (Tra.) The antigen has returned completely to the nucleus of one cell but it is still partly outside the nuclear membrane of the other cell x1500.

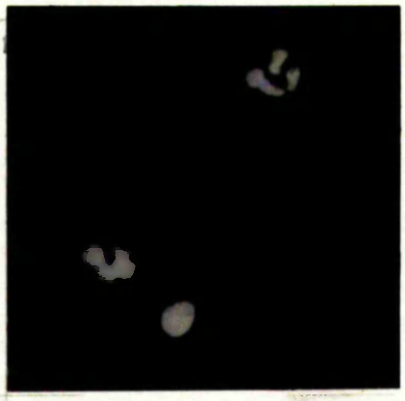


Fig. 11/1 Normal blood film: polymorphs and lymphocyte nuclei stained with "homogeneous" antinuclear serum (War.) x850.

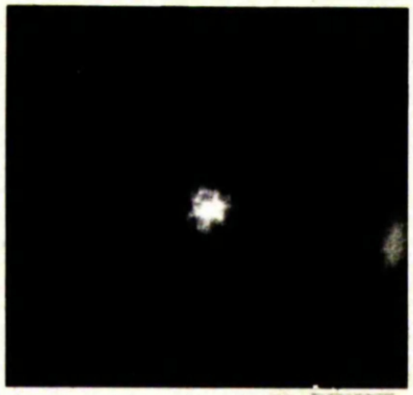


Fig. 11/2 Normal blood film: lymphocyte nucleus stained with "speckled" antinuclear serum (Ral.) x1200.

Fig. 11/3 Normal blood film: monocyte nucleus stained with "speckled" antinuclear serum (Ral.) x1200.

Fig. 11/4 Normal blood film: polymorph nucleus not stained with "speckled" antinuclear serum (Ral.) x1500.

Fig. 11/5 Normal blood film: lymphocyte stained with antinucleolar serum (Pac.) to demonstrate 1 nucleolus x1200.

Fig. 11/6 Normal blood film: monocyte stained with antinucleolar serum (Pac.) to demonstrate 3 nucleoli x1200.

Fig. 11/7 Normal blood film: polymorph nucleus not stained with anti-nucleolar serum (Pac.) x800.

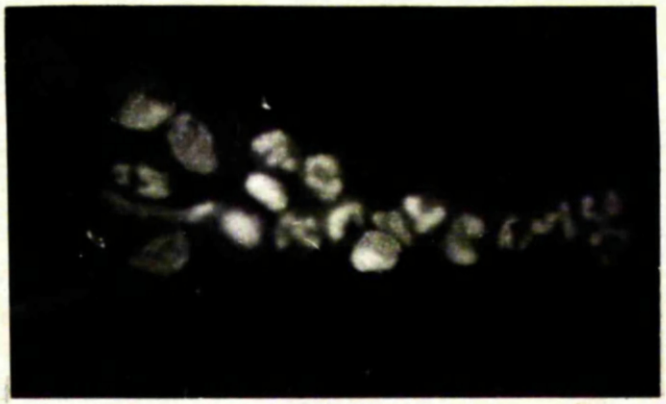


Fig. 11/8 Chronic myeloid leukaemia blood film stained with "homogeneous" antinuclear serum (Dun.) x550.

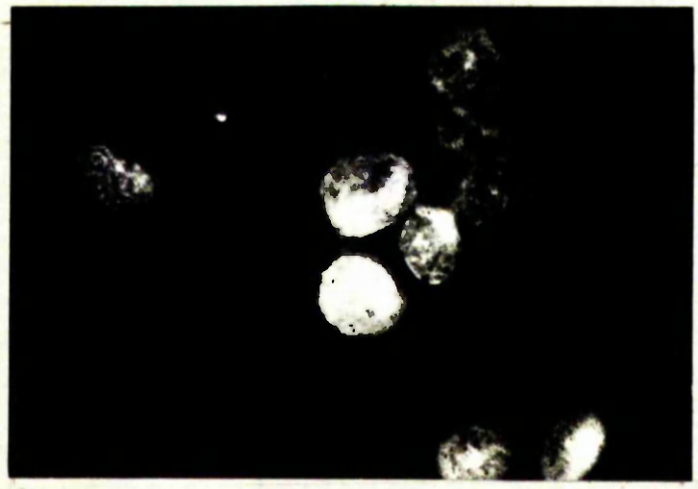


Fig. 11/9 Chronic myeloid leukaemia blood film stained with "speckled" antinuclear serum (Ral.). Nuclear staining is more intense in the nuclei of the less well differentiated cells x1200.

Fig. 11/10 Chronic myeloid leukaemia stab cell stained with "speckled" antinuclear serum (Tra.) showing staining restricted to one limb of the nucleus x1200.

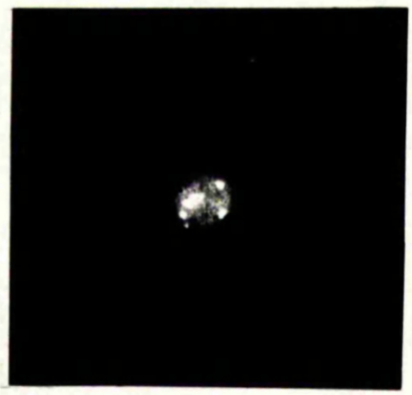


Fig. 11/11 Chronic myeloid leukaemia myelocyte stained with antinucleolar serum (Mui.) to demonstrate 5 nucleoli x1200.

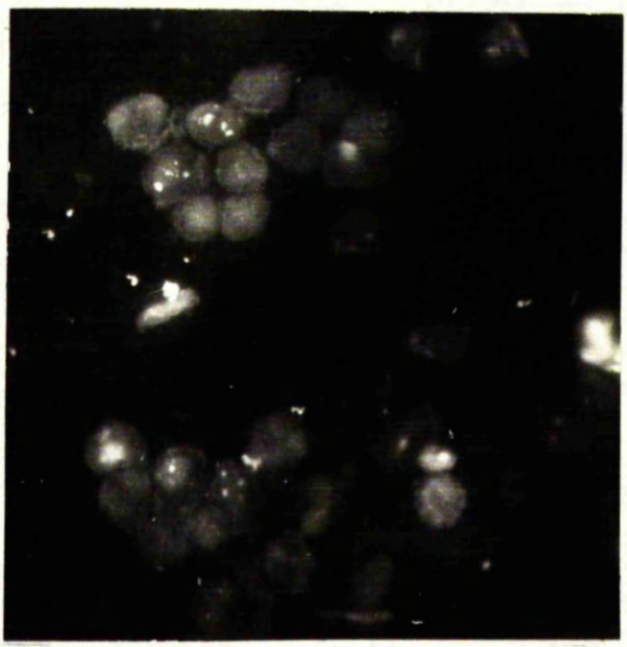


Fig. 11/12 Chronic myeloid leukaemia blood film stained with antinucleolar serum (Pac.) x550.

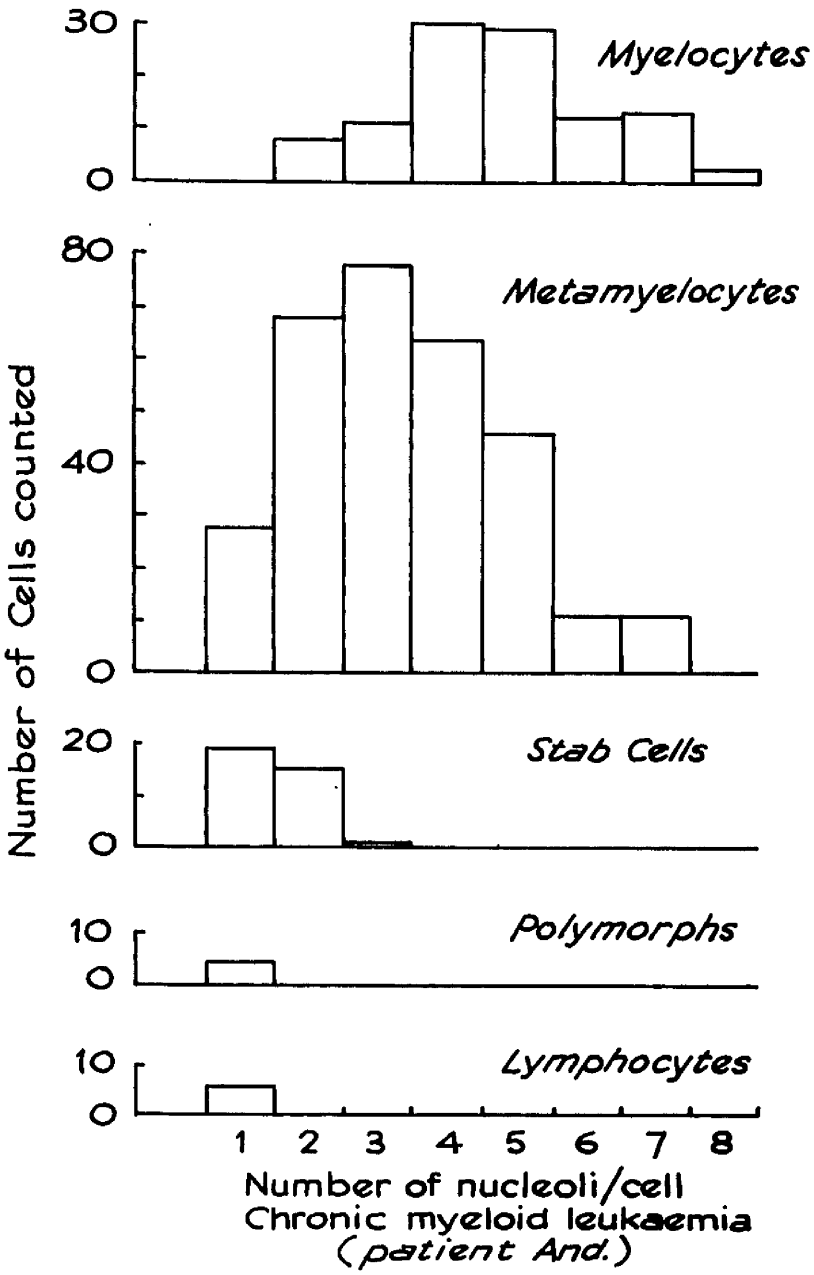


Fig. 11/13 Number of nucleoli in the nuclei of different cell types in blood films from chronic myeloid leukaemia patient (And.)

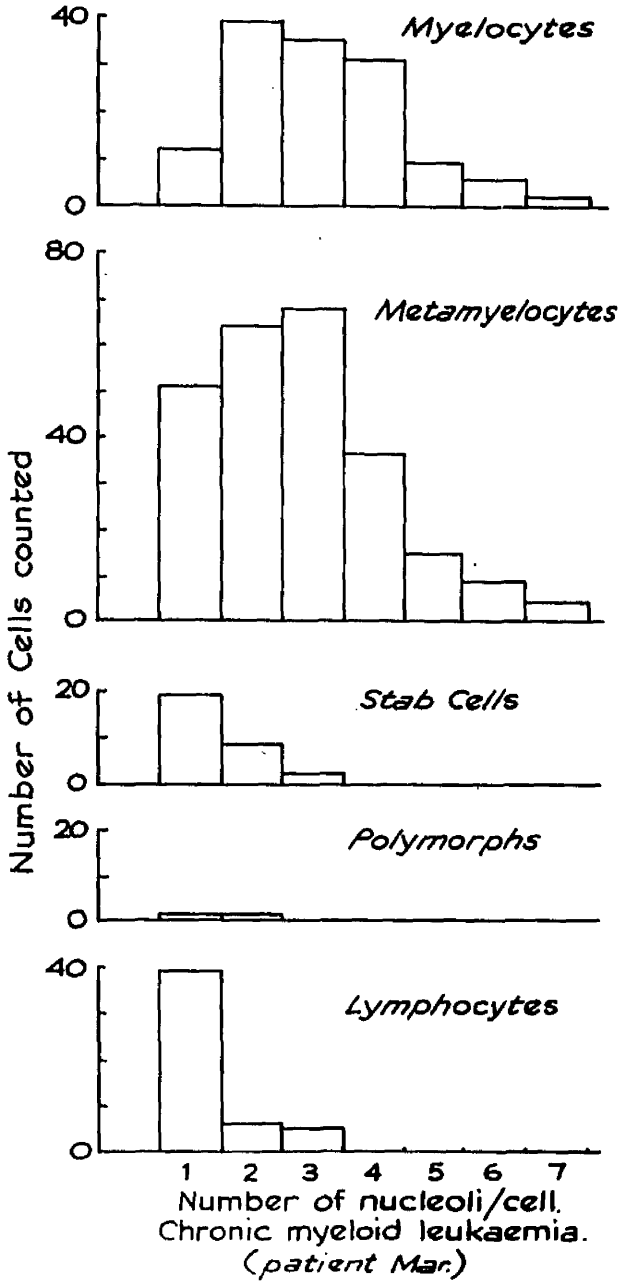


Fig. 11/14 Number of nucleoli in the nuclei of different cell types in blood films from chronic myeloid leukaemia patient (Mar.)

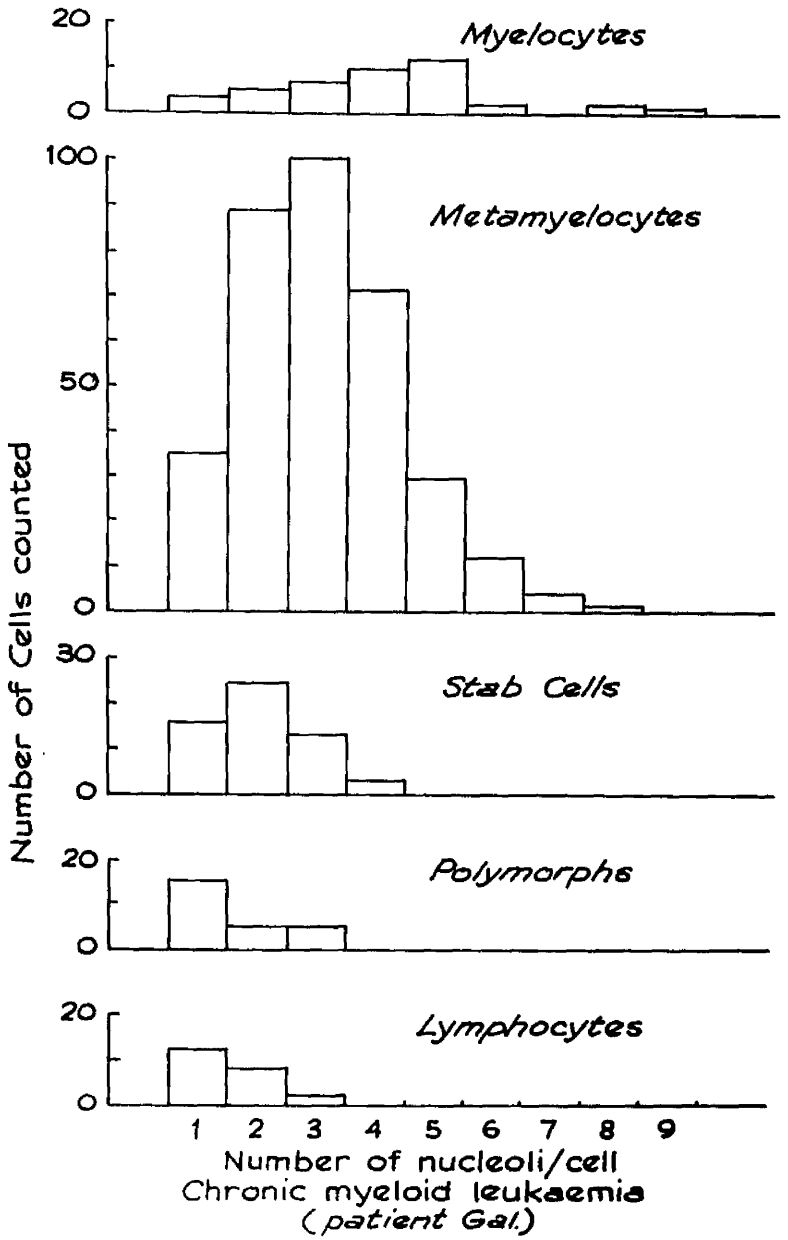


Fig. 11/15 Number of nucleoli in the nuclei of different cell types in blood films from chronic myeloid leukaemia patient (Gal.)

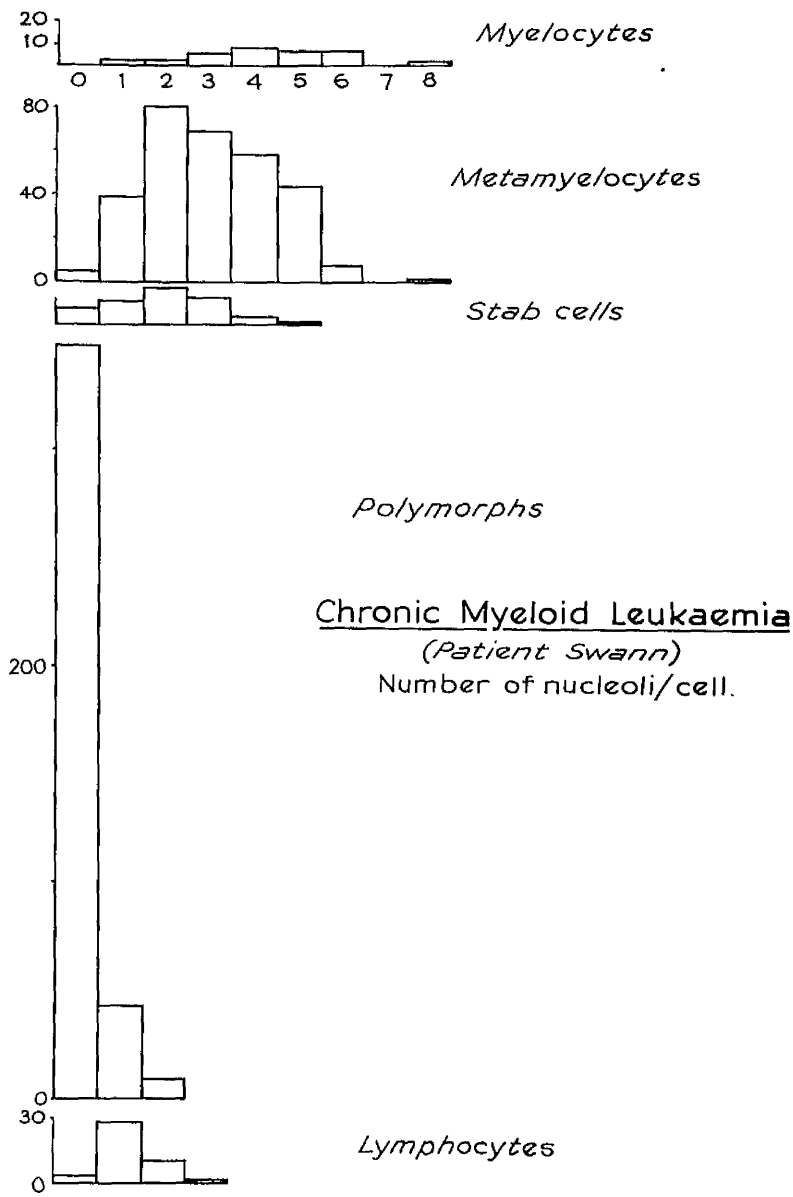


Fig. 11/16 Number of nucleoli in the nuclei of different cell types in blood films of chronic myeloid leukaemia patient (Swa.)

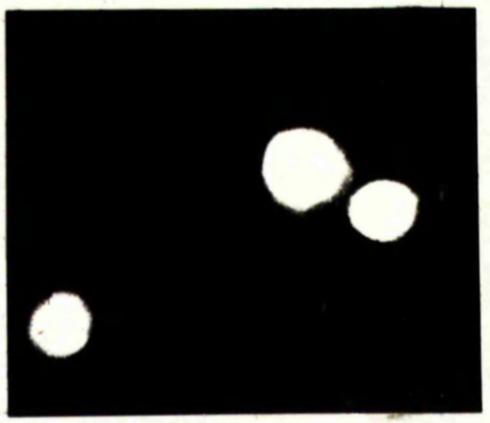


Fig. 11/17 Chronic lymphatic leukaemia blood film stained with "homogeneous" antinuclear serum (War.) x1200.

Fig. 11/18 Chronic lymphatic leukaemia blood film: lymphocyte stained with antinuclear serum (Pac.) to demonstrate 1 nucleolus x1200.

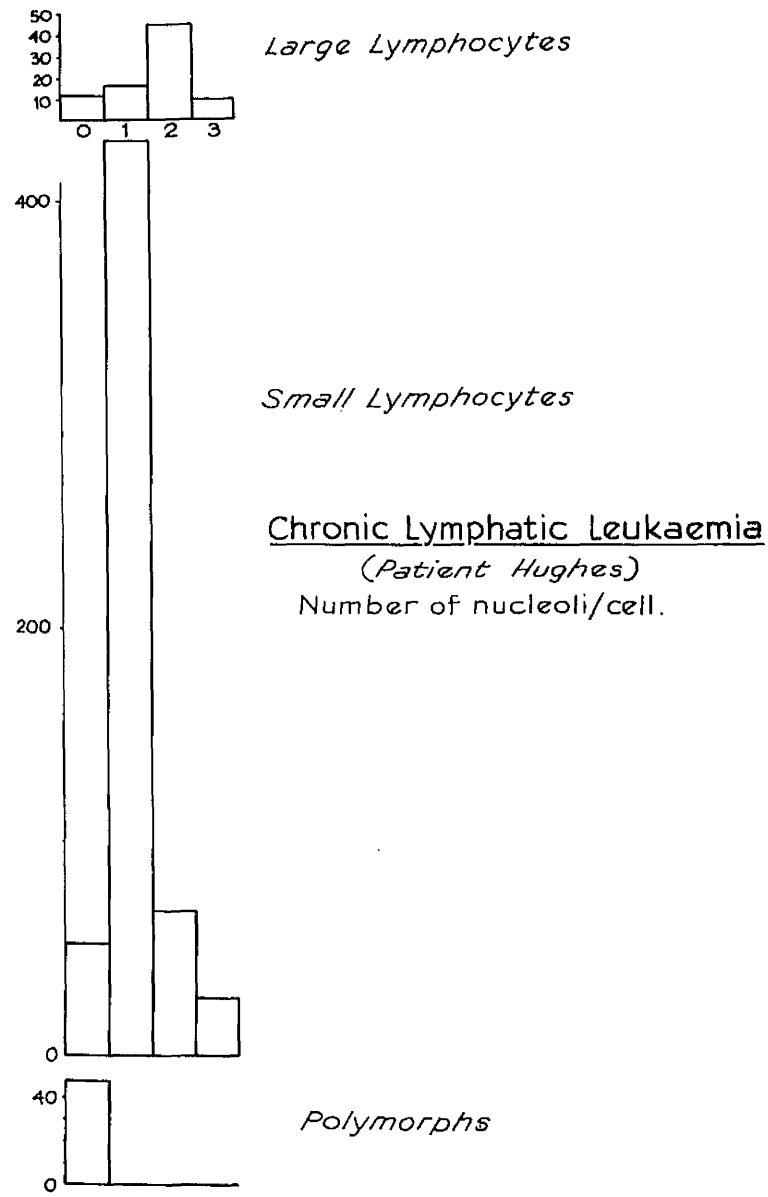


Fig. 11/19 Number of nucleoli in the nuclei of different cell types in blood films of chronic lymphatic leukaemia patient (Hug.)

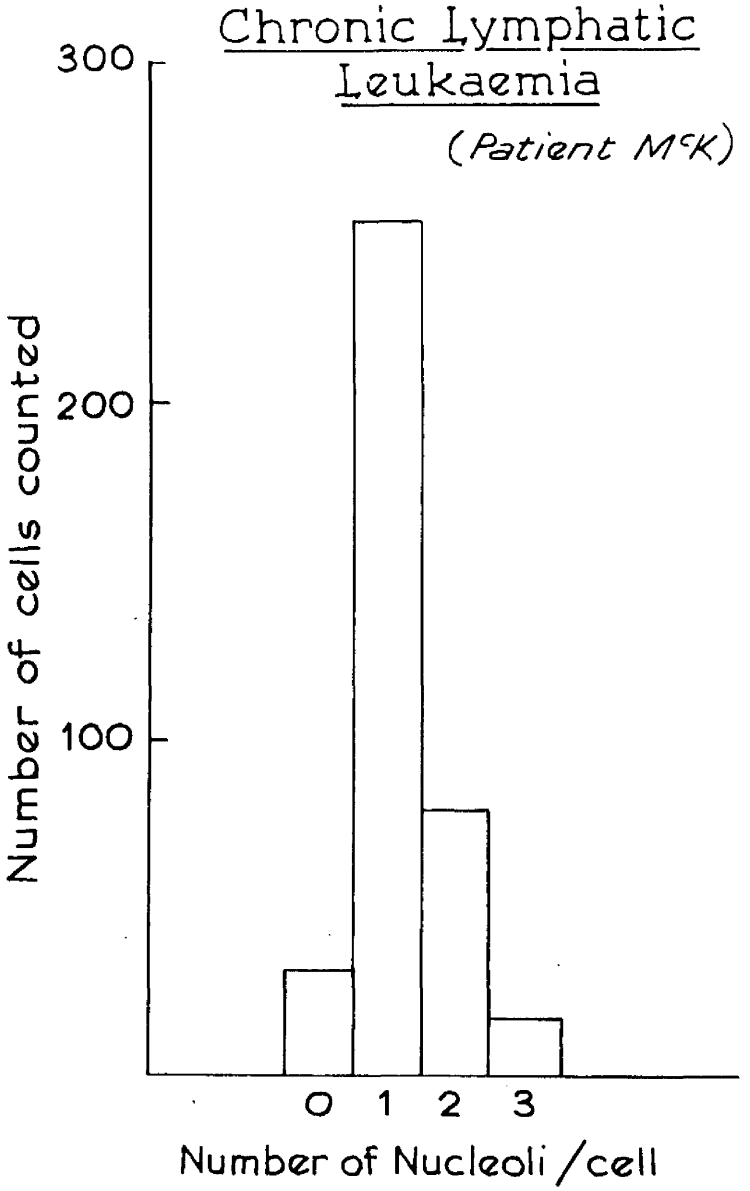


Fig. 11/20 Number of nucleoli in cell nuclei in blood films of chronic lymphatic leukaemia patient (McKen.)

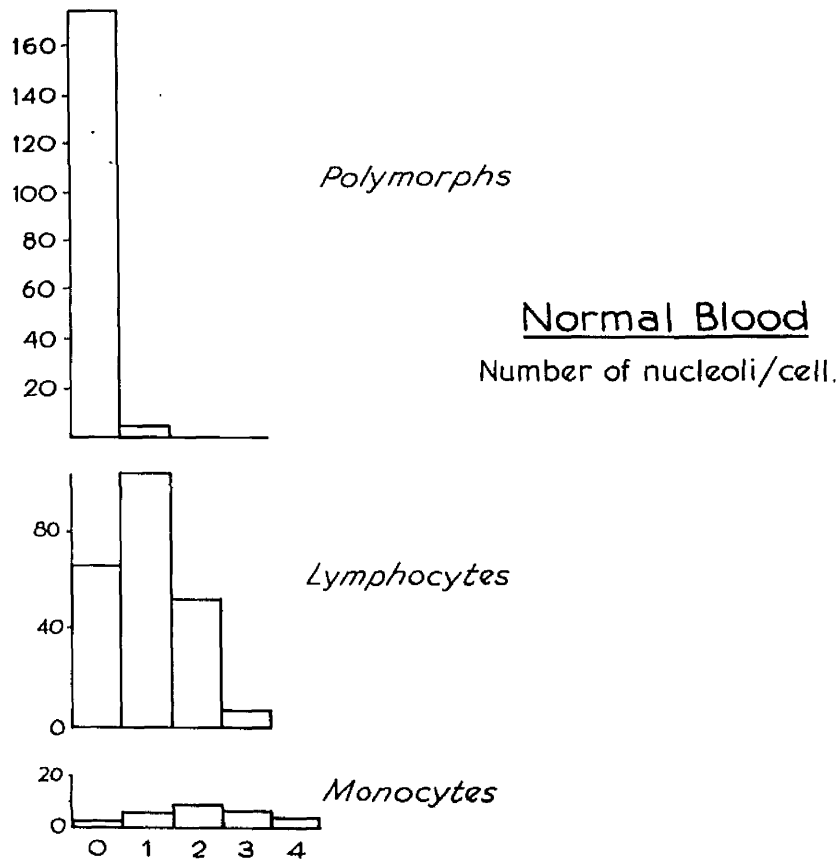


Fig. 11/21 Number of nucleoli in the nuclei of different cell types in normal blood films (Bec.)

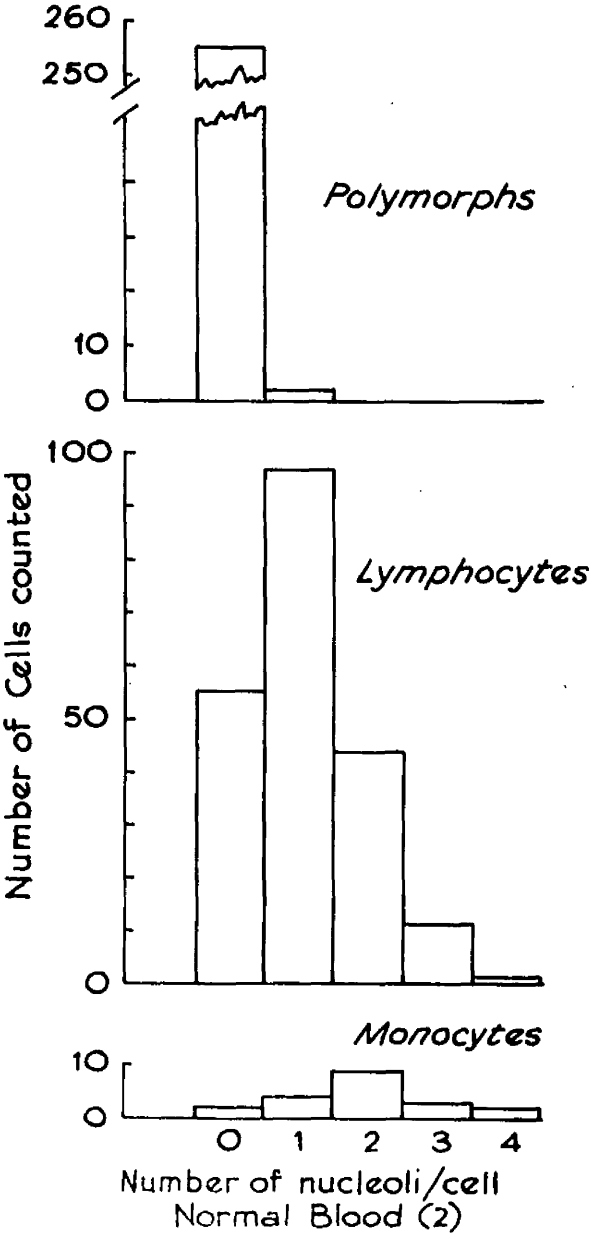


Fig. 11/22 Number of nucleoli in the nuclei of different cell types in normal blood films (Cam.)

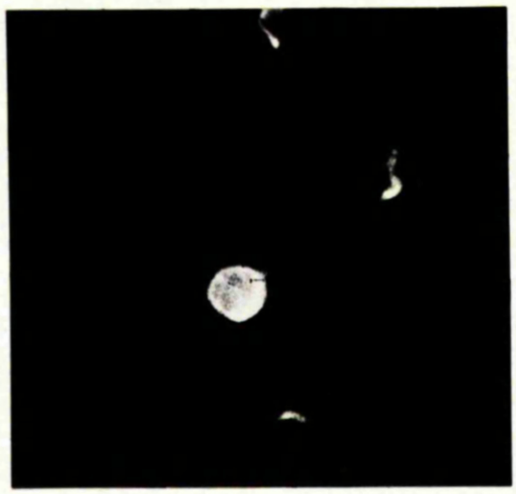


Fig. 12/1 Infected rat blood film: lymphocyte and Trypanosoma gambiense nuclei stained with anti-DNA (McDou.) x1200.

Fig. 12/2 Infected rat blood film: lymphocyte nucleus stained with anti-nucleohistone serum (War.), but T. gambiense nucleus unstained. x1200.

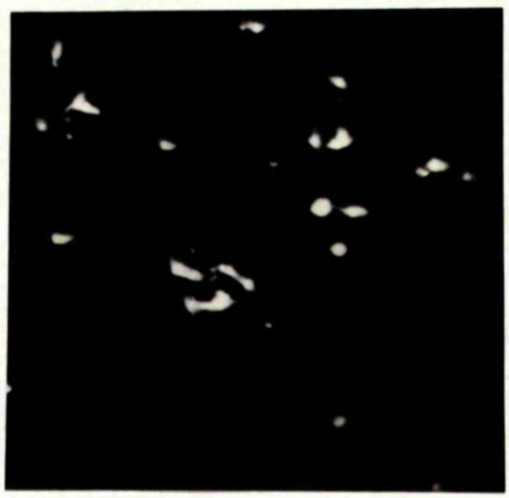


Fig. 12/3 T. rhodesiense culture: nuclei stained with anti-DNA (McDou.) x850.

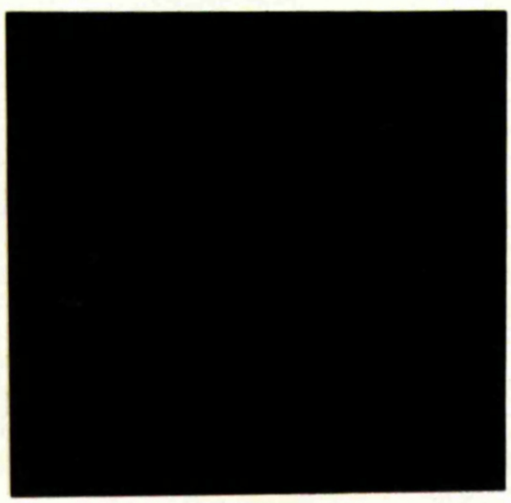


Fig. 12/4 T. rhodesiense culture: nuclei unstained by anti-nucleohistone (War.) x850.

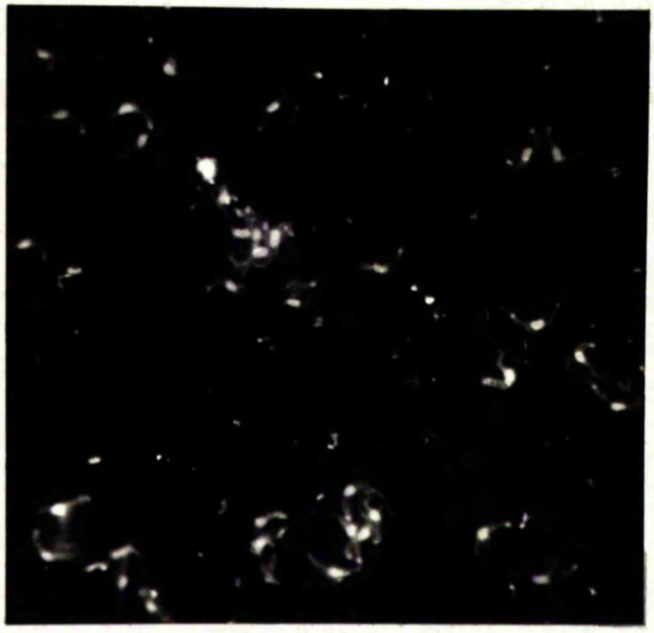


Fig. 12/5 *T. cruzi* infected rat blood film: nuclei stained with anti-DNA (McDou.) x550.

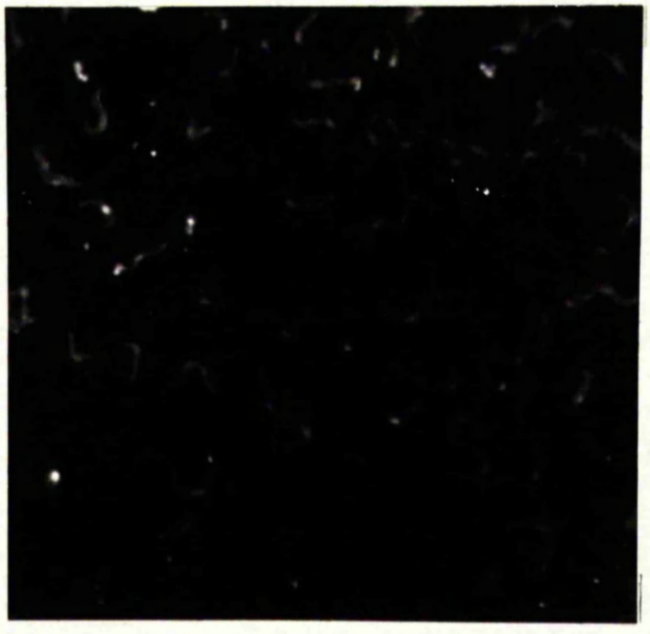


Fig. 12/6 *T. cruzi* infected rat blood film: nuclei unstained with anti-nucleohistone (War.) x550.

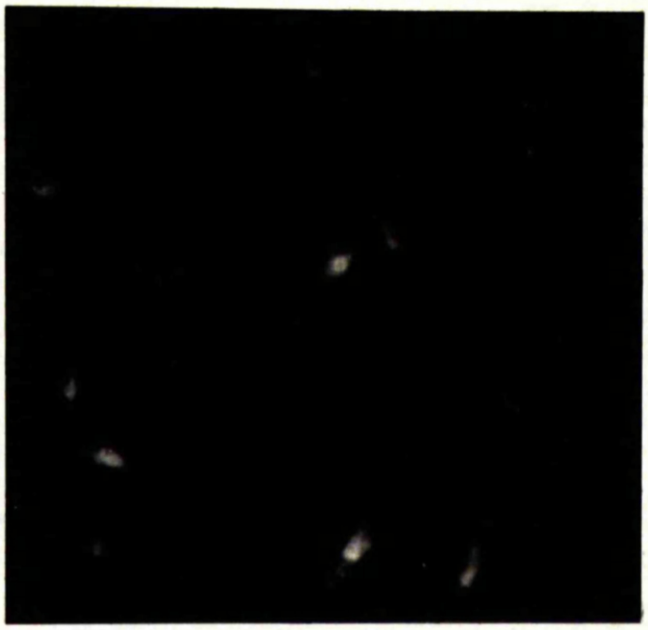


Fig. 12/7 *T. cruzi* culture: nuclei stained with anti-DNA (McDou.) x850.

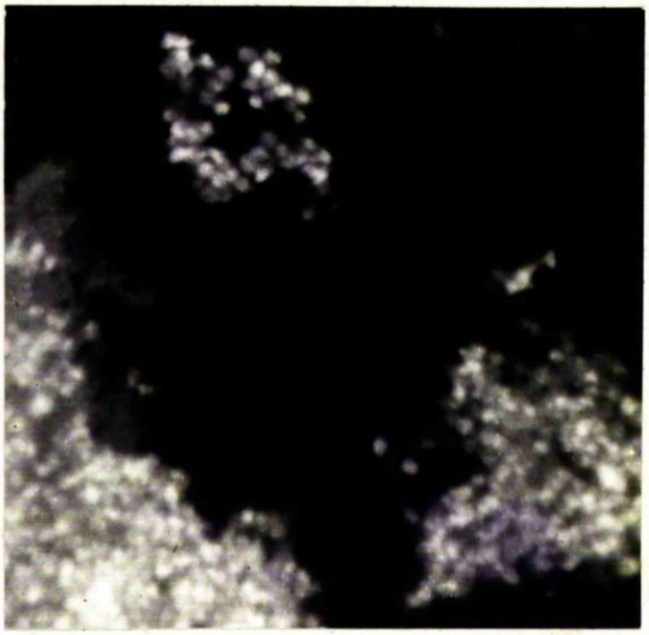


Fig. 12/8 *T. lewisi* culture: nuclei stained with anti-DNA (McDou.) x550.

Fig. 12/9 *T. congolense* culture: nuclei stained with anti-DNA (McDou.) x550.

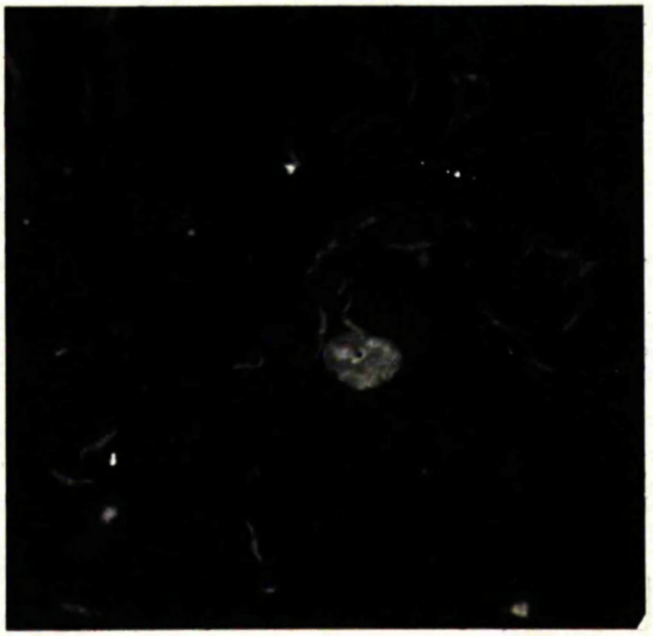


Fig. 12/10 *T. congolense* infected rat blood film: polymorph nucleus stained by anti-nucleohistone (War.) whereas trypanosome nuclei unstained x850.

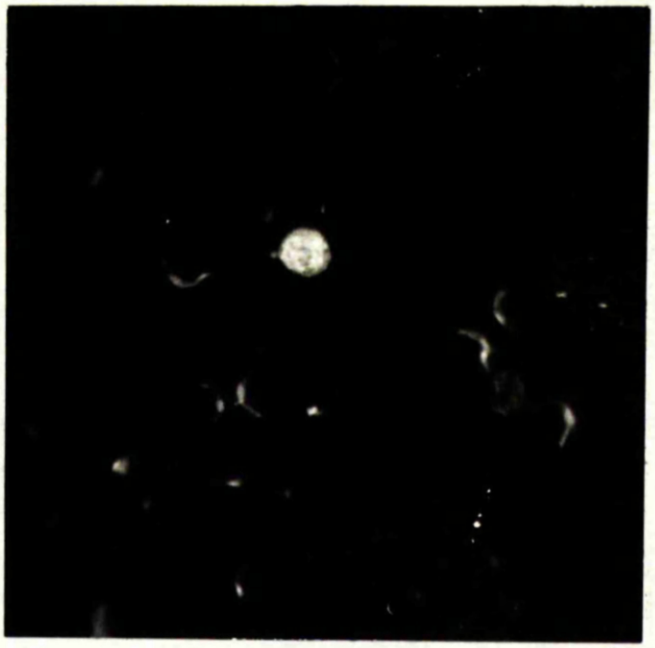


Fig. 12/11 *T. evansi* S.A.K. infected rat blood film: trypanosome and leucocyte nuclei stained with anti-DNA (McDou.) x850.

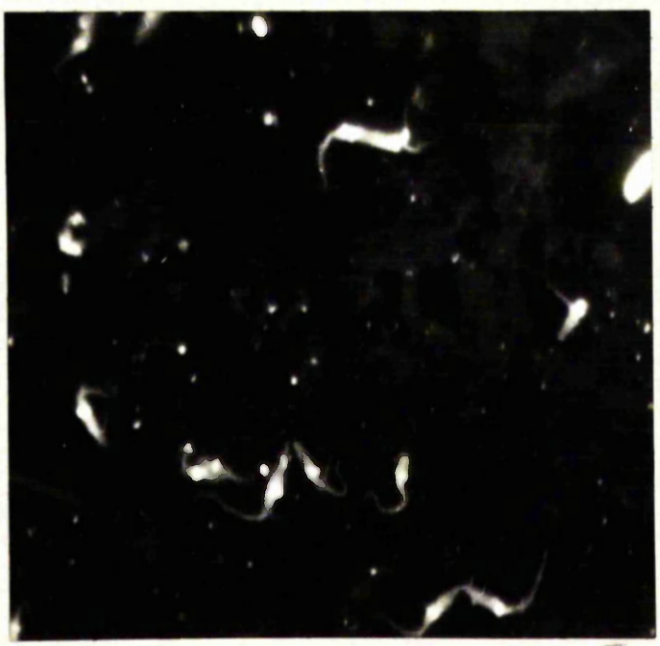


Fig. 12/12 *Leishmania donovani* culture: nuclei stained with anti-DNA (McDou.) x850.

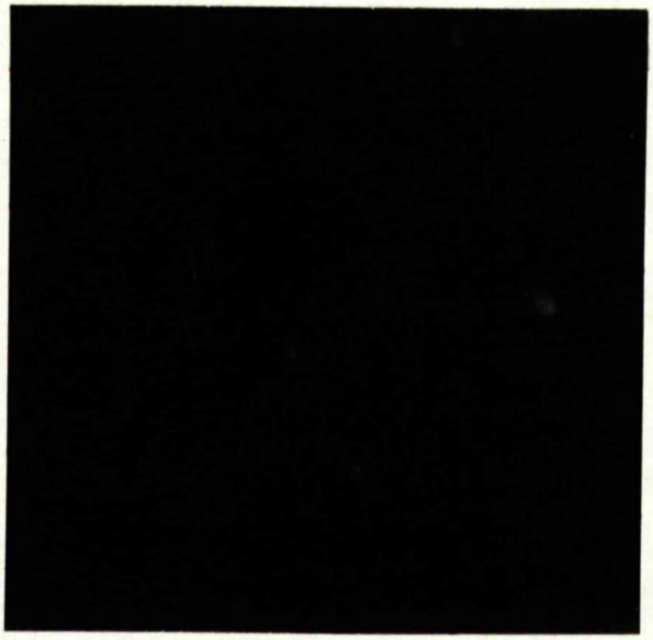


Fig. 12/13 Leishmania donovani culture: nuclei unstained by anti-nucleohistone (War.) x850.

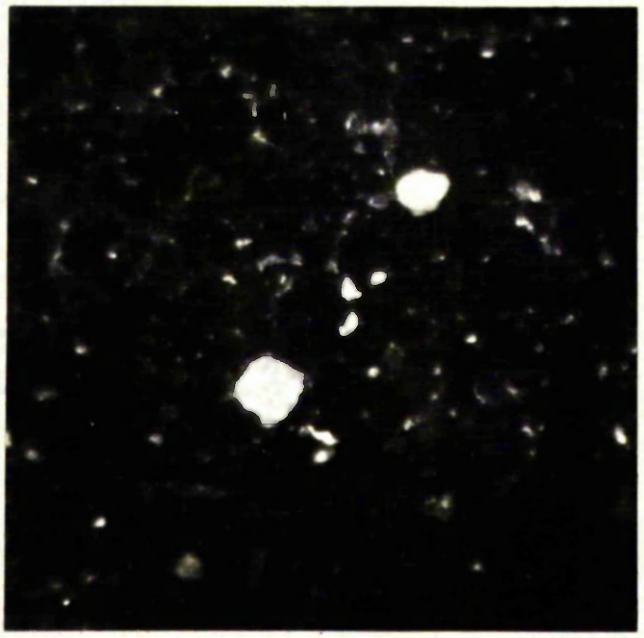


Fig. 12/14 Toxoplasma gondii infected mouse peritoneal exudate: leucocyte and parasite nuclei stained with anti-DNA (McDou.) x850.

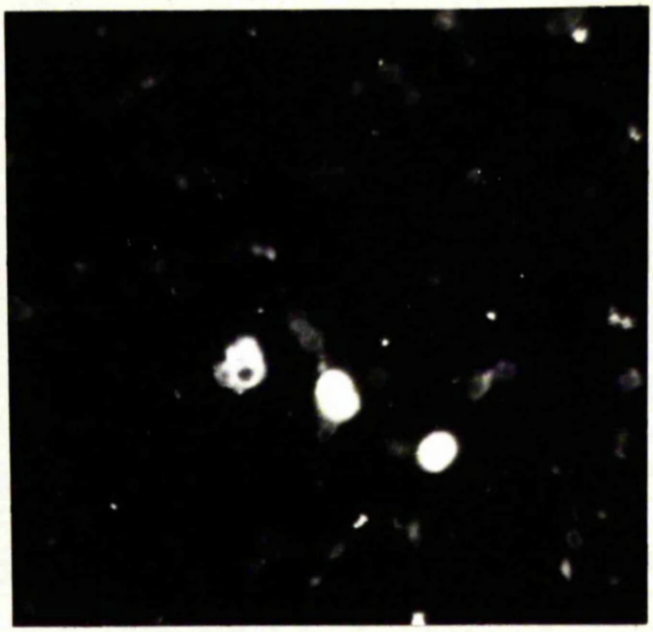


Fig. 12/15 *Toxoplasma gondii* infected mouse peritoneal exudate: leucocyte nuclei stained by anti-nucleohistone (War.) whereas parasite nuclei unstained x850.

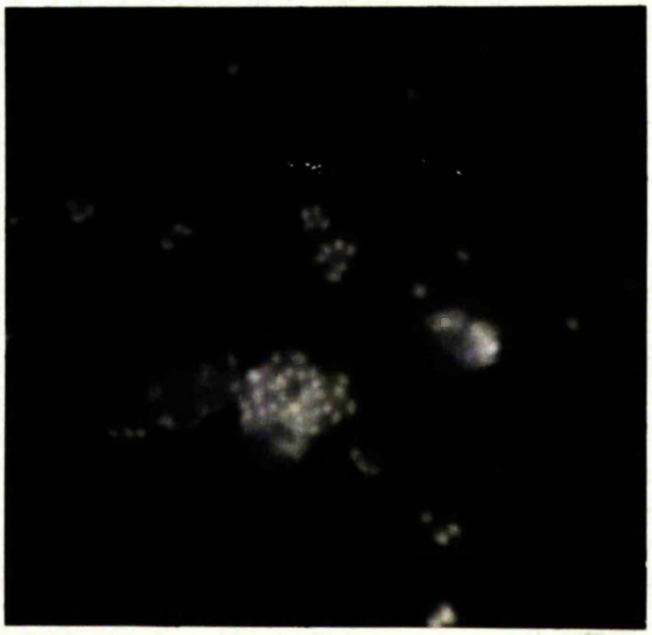


Fig. 12/16 *Toxoplasma gondii* concentrate from infected mouse peritoneal exudate: nuclei stained with anti-DNA (McDou.) x550.

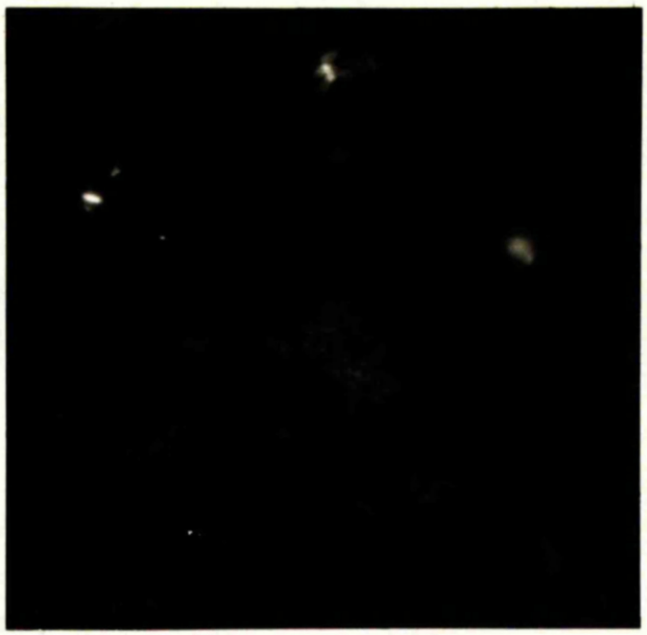


Fig. 12/17 *Toxoplasma gondii* concentrate from infected mouse peritoneal exudate: nuclei unstained by anti-nucleohistone (War.) x550.

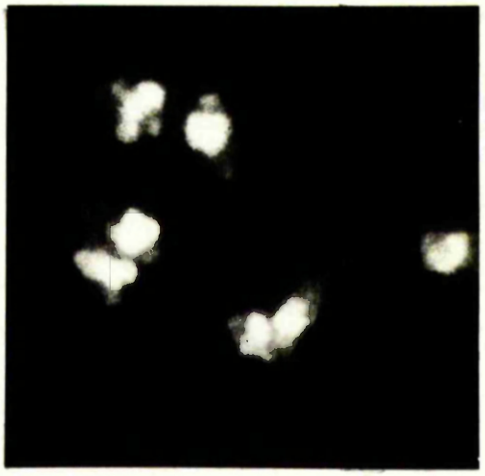


Fig. 12/18 Strigomonas Sp. culture: nuclei stained with anti-DNA (McDou.) x1000.

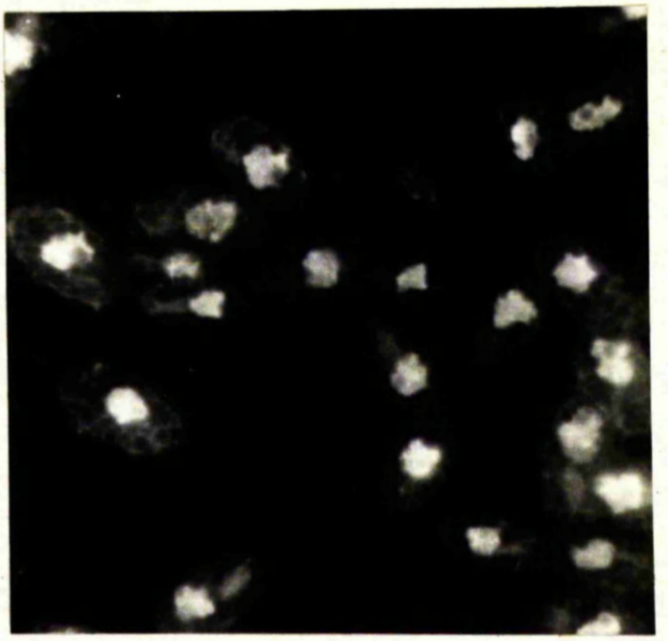


Fig. 12/19 Peranema Sp. culture: nuclei stained with anti-DNA (McDou.) x550.

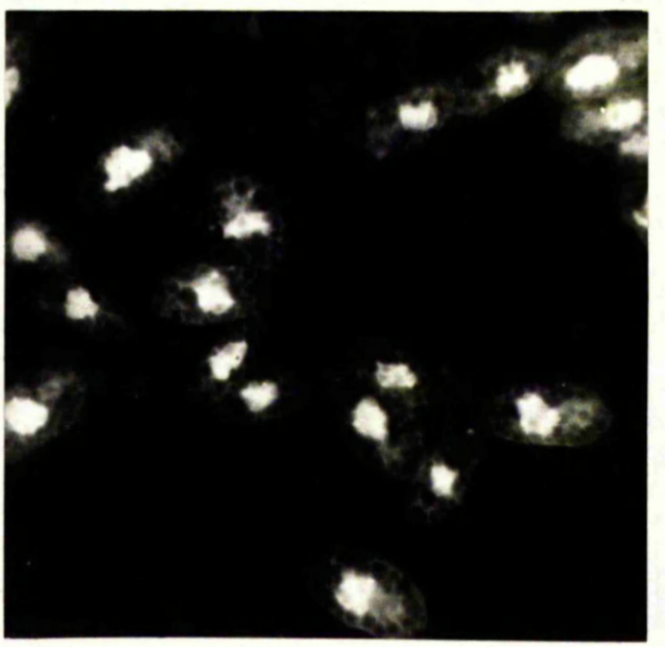


Fig. 12/20 Peranema Sp. culture: nuclei stained by anti-nucleohistone (War.) x550.

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