THE ANTI-NUCLEAR FACTOR AS AN AUTO-ANTIBODY

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PREFACE

It is interesting to ponder the reason for the flowering of interest, observation and experimental activity centred on the notion of auto-immunity, observed over the past decade. An important element in this development must be attributed to the refinements in immunological techniques for the detection and study of antibody, not the least among which was the observation of Coons et al. (1953) that tissue antibody can be identified specifically by an immunofluorescence sandwich technique and by White (1954) of the antibody nature of Russell bodies experimentally produced in rabbit spleen cells. These observations were the prelude to an important series of immunofluorescence studies reported in 1957 and 1958 showing anti-thyroid antibodies (White 1957), auto-antibodies to nervous tissue in experimental animals (Beutner et al. 1958) and the simultaneous report from three different laboratories of anti-nuclear activity in Systemic Lupus Erythematosus (Friou 1957, Holborow, Weir and Johnson 1957, and Holman and Kunkel 1957).

Since this time, a flood of reports of different aspects of anti-nuclear activity have been published, the last in September 1962. The implications of an antibody to the ubiquitous nuclear material

seemed immense. The nuclear antigens which appeared to be concerned were not included in the category of substances known to be antigenic. With the support of a Fellowship from the Empire Rheumatism Council, the author undertook a study of the anti-nuclear factor during the period June 1957 to August 1960 under the supervision of Dr. E.J. Holborow and Dr. L.E. Glynn. The author wishes to acknowledge the advice and constant encouragement of both these colleagues and the interest and support of Professor E.G.L. Bywaters in whose Rheumatism Research Unit the work was carried out. Thanks are due to the following:- Dr. D. Doniach, Dr. W. Hijmans, Dr. A. Scott, Dr. E.G. Rees, Dr. B. Ansell, Dr. Dölle and Dr. A. Dixon who made this study possible by providing specimens of sera and clinical information on their patients; Dr. R. Consden who advised and assisted in biochemical problems; Mr. P. Fiske and Mr. T.C. Dodds who prepared the plates and figures; and Mrs. G. McCallum who typed the manuscript. Professor R. Cruickshank kindly read the manuscript and gave valuable criticism.

The results of some of the work incorporated in this thesis have been published, as noted in the text. Other relevant references not quoted are:- Weir, D.M. and Holborow E.J. 1962. Ann. Rheum. Dis. 21, 40. and Holborow E.J. and Weir D.M. 1959. Lancet, 1, 809.

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INTRODUCTION

The classic studies of Ehrlich and Morgenroth carried out at the beginning of this century were of the greatest importance in investigating the ability of the individual to react immunologically against its own body material. In their third communication on haemolysins (1900) they state "In pathology the changes foremost to be considered are those resulting from the absorption, by an organism, of its own cell material. Such occasions are presented by many different diseases." "If an individual suffers a considerable subcutaneous haemorrhage or one into a body cavity, or if part of his blood corpuscles are destroyed and lysed by certain blood poisons, the essential conditions, just as in experiment, are given for the formation by reaction of substances possessing specific injurious affinities for these blood cells." Later in the same communication they point out that "it is of the highest pathological importance to determine whether the absorption of its own body material can excite reactive changes in the organism, and what the nature of these changes is." Accordingly, Ehrlich and Morgenroth immunised a "strong male goat" with nearly a litre of blood obtained from three other goats. The serum which they obtained following immunisation lysed the cells of all but one of nine goats they tested. Significantly the cells of the immunised goat itself were completely unaffected by the haemolytic serum.

Ehrlich explained this phenomenon by means of his side chain theory of antibody production and proposed that an anti-autolysin may be produced in the following manner:- If only small amounts of antibody (the receptor liberated from cells by antigen) reach the animals own red cells, this would effect a production and thrusting off of the corresponding receptor from the red cells, which "would then circulate as an anti-autolysin and serve to switch the autolysin thereafter formed away from the blood cells".

Further investigation carried out by Ehrlich convinced him of the existence of "certain contrivances by means of which the immunity reaction, so easily produced by all kinds of cells, is prevented from acting against the organism's own elements and so giving rise to autotoxins" (Ehrlich and Morgenroth 1901). This led Ehrlich to enunciate his doctrine of "Horror autotoxicus". This concept still stands today, and the breakdown of the "internal regulating devices" now termed the "homeostatic mechanism" by Burnet has been shown to lead to the great dangers to the individual conceived of by Ehrlich. About the same time that Ehrlich and Morgenroth were studying the production of haemolysins, Metalnikoff (1900),working in the laboratory of Metchnikoff, showed that guinea-pigs immunised by the intra-peritoneal injection of sperms from another guinea-pig produced a "spermotoxine" which would kill in vitro not only the sperms of other guinea-pigs, but also those of the animal which had been immunised. It is interesting to note that

Ehrlich was unable to accept this "auto-spermotoxine" as an auto-toxin (i.e. involving a breakdown of the internal regulating devices), as the sperms were not killed in vivo as required by his definition. Time has shown that Ehrlich was right (although for the wrong reasons) in that antibody production to sperms does not constitute a breakdown of the internal regulating devices controlling antibody production, but is due to the "foreign nature" of sperms resulting from their usual isolation from the antibody forming cells ("hidden antigen").

At that time a number of reports indicating the existence of auto-immune phenomena appeared. Thus the first recognisable description of acquired haemolytic anaemia, generally credited to Hayem in 1898, was followed by the finding of abnormal haemolysins in patients in whom intense haemolysis was taking place in vivo (Chaufford and Troisier 1908, Chaufford and Vincent 1909). In 1904 the phenomenon of paroxysmal cold haemoglobinuria was described by Donath and Landsteiner. The work of Bergmann and Savini in 1907 suggested a disturbance of the internal regulating mechanism when they found antibodies to liver in rabbits poisoned with phosphorus. However, such was the influence of Ehrlich that despite the appearance of these reports, the scepticism aroused by him prevented the recognition of their significance. These pioneer studies were neglected for about 30 years until Damashek and Schwartz in 1938 reported the finding of abnormal haemolysins in patients suffering from acute acquired haemolytic anaemia.

The study of the serological manifestations of these phenomena over the next few years was greatly aided by the introduction of a technique utilising anti-globulin sera (Coombs et al. 1945). The "direct anti-globulin reaction" (Coomb's test), which enabled the detection of non-agglutinating antibody attached to red cells, was applied to the investigation of cases of haemolytic anaemia, and in 1946 Boorman et al. reported that this reaction was positive in a number of patients suffering from "idiopathic" acquired haemolytic anaemia. They were thus able to distinguish these cases from the congenital form of the disease. During the next decade, the antibodies of acquired haemolytic anaemia were extensively studied and the concept of auto-immunisation in this condition became widely accepted.

The antibodies found in auto-immune haemolytic anaemia are divided on the basis of their laboratory behaviour into two main groups: the "warm antibodies" and the "cold antibodies". The warm type are gamma globulins with a molecular weight similar to that of antibody gamma globulin (160,000), and likewise have a sedimentation constant of 7 S. in the ultracentrifuge. These antibodies sensitise erythrocytes to agglutination by anti-globulin serum and are not themselves able to agglutinate normal red cells. They are active at 37°C. and rarely, if ever, lyse normal cells at this temperature (Dacie 1962). In contrast, the cold antibodies are markedly potientated in activity by fall in

temperature, and are not usually active at 37°C. They are powerful haemagglutinins and will lyse the cells of patients with paroxysmal cold haemaglobinuria and enzyme treated normal cells. These antibodies are high molecular weight 19 S. globulins. An essential feature of the antibodies of acquired haemolytic anaemia is their ability to act on the patient's own erythrocytes. Until 1953 these antibodies had been generally considered to be "non-specific" in that they would react with antigens present on all human erythrocytes. It is now known that these auto-antibodies frequently show a definite specificity usually within the Rh system (Dacie 1962). Dacie concludes that the blood group specificity of these antibodies by virtue of which they are indistinguishable from blood group iso-antibodies justifies the acceptance of these globulins as antibodies without quotation marks.

Associated with the accumulation of evidence regarding the auto-immune nature of the haemolysins in acute haemolytic anaemia, there arose the need to consider the meaning of foreignness and self-recognition and the ability of the body to differentiatie between "self" and "non-self". A solution to the problem this created in immunological theory was offered by Burnet and Fenner in 1949. At that time antibody production was envisaged in terms of the antigen acting as a template for modifying the globulin molecules to a form which was complementary to that of the antigen. It was considered that antigen

may act either on the already formed protein chain and bring about a reconfiguration of the chain (Pauling 1940), or that the antigen induced a chemical re-arrangement of the globulin molecule as it was formed (Mudd 1932). Burnet and Fenner, whilst accepting this direct template theory, included the notion of "self markers" attached to body components by which antibody-forming cells were able to recognise them, thus rendering them immunologically inert. They also predicted that an equivalent tolerance to foreign antigens should be demonstrable if these had been introduced at an appropriate stage in embryonic life. This prediction was later confirmed experimentally by Medawar and his colleagues (Billingham, Brent and Medawar 1956). The persistence of antibody-producing capacity after antigenic stimulation was explained by the notion that genetic modification of the antibody-producing cell was induced by the antigen, so allowing the indefinite production of descendent antibody-producing cells.

In 1955, an alternative hypothesis which did not require the "self marker" postulate was formulated by Jerne in his natural selection theory in which, for the first time, natural antibodies were seriously considered in relation to 'true' antibodies. It postulated that a comprehensive range of gamma globulin molecules existed in the serum, comprising carriers of all the reactive sites needed to unite with any potential antigenic determinant, except those already existing in accessible components of the body. The function of the antigen which

enters the body from without is to act as a selective carrier of spontaneously circulating antibody to a system of cells which can reproduce this antibody. It is assumed that once antibody is taken into the cells of the antibody-producing system, replicas of this natural antibody will be produced. Influenced by Jerne's ideas, and by current concepts of adaptive enzyme formation which no longer accepted that a genetic change occurred following exposure to the inducer but that the pattern of the adaptive enzyme had already been genetically determined, Burnet formulated his Clonal Selection Theory. This theory allowed the antigen no part in impressing pattern on the antibody-producing cell. The capacity to produce a given antibody was regarded as a genetically determined quality of certain clones of mesenchymal cells, the function of the antigen being to stimulate cells of these clones to proliferation and antibody production. The theory postulated that in the early stages of embryonic development during the differentiation of mesenchymal cells, the genetic control of globulin production was temporarily relaxed (i.e. there was a temporary phase in which the mesenchymal cells showed a high degree of somatic mutability). This led to a large variety of random arrangements in the globulin molecules produced. When control was re-established, these random sequences were permanently adopted by the cells concerned (i.e. a very large number of clones were generated which would correspond to potential antigenic determinants). The theory made the further assumption that an

immunologically competent cell, if embryonic, would react with the corresponding antigenic determinant and be destroyed, whilst if adult it would be stimulated to proliferate. This assumption was required to explain how cells which had undergone the unfortunate mutation of being able to react to self antigens were eliminated. A simple hypothesis which would provide such a homeostatic mechanism was to assume that once a cell had been stimulated to proliferate by an antigenic stimulus, it would pass through a transient phase in which it was susceptible to destruction or inhibition on further contact with the same antigenic determinant. Whenever the antigenic determinant was freely present in the body and accessible to the corresponding immunologically competent cells, such destructive contacts would be inevitable. Tolerance would result from the elimination or inhibition of all cell clones which reacted with antigenic determinants present in the body. After birth when foreign antigen entered the body antibody to it would be produced by specific stimulation of those cells in clones pre-adapted to react with the corresponding antigenic determinants. On the basis of this theory, Burnet accounted for the development of auto-immune disease as a partial failure and often temporary failure of homeostatic mechanism.

Before proceeding to detail further the auto-immune phenomena which the above theories were attempting to account for, it is necessary to mention some of the techniques which made their study possible. The anti-globulin test of Coombs already referred to was one of the most valuable. Its use was extended to the study not only of haematological phenomena, but also to detect the uptake of globulin by a variety of other tissue cells - the so-called "anti-globulin consumption test". The anti-globulin technique became a valuable adjunct to the fluorescent protein tracing technique of Coons and Kaplan (1950). Its incorporation into this technique enabled the visualisation in a tissue or cell preparation examined under the fluorescence microscope, of antibody globulin shown up by staining the preparation with fluorescein-conjugated anti-globulin serum. Fluorescent protein tracers could be detected in animal tissues by fluorescence microscopy at a concentration of the order of 1 ug. protein per ml. of body fluid (Nairn 1962), which was more than a hundredfold better than with non-fluorescent dye labels such as Evans blue or other azo-dyes. Fluorescein isocyanate was used in this technique until 1958 when Riggs et al. introduced the more stable isothiocyanate which could be conjugated with protein in a much simpler manner and which could be obtained commercially. It was shown that fluorochrome dyes combined with the free amino groups of the lysine side chains, with other free amino groups and with free carboxyl groups both at the end of each protein chain and in the aspartic and glutamic

acid residues. Fluorescein isothiocyanate was shown to absorb light in the ultra-violet region with absorption peaks at 290, 325 and 495 mu (Nairn 1962) and to emit blue-green light with a wavelength of 520 mu.

Other valuable procedures involved the elaboration of the classical techniques of precipitation and agglutination. The technique of immunodiffusion allowed precipitation to take place on gels and other forms of support media, and provided an analytic technique of high resolution and specificity. The technique of immunoelectrophoresis, described by Grabar and Williams, (1953) was a further development which used preliminary electrophoresis in agar to give separation of the individual components and allow the easy identification of precipitin bands. The agglutination phenomenon was extended to include the use of non particulate soluble antigens in the reaction. This was achieved by the use of tannic acid treated red cells, or the inert particles polystyrene latex, bentonite and collodion. These particulate materials when coated with the soluble antigen were shown to become susceptible to agglutination by the appropriate antibody. Finally, in recent years, the technique of cell or tissue transfer has found increasing application as an approach in the study of immunological problems. This method essentially involves the transfer of tissues from one animal, where some immunological process has begun to a normal animal of the same species, in which further immunological developments can be observed.

Among the diseases in which an auto-immune basis is widely accepted is human thyroiditis. It has been shown experimentally in rabbits, dogs and guinea pigs that intradermal injection of thyroid extract (combined with Freund's complete adjuvant) from other animals of the same species results in the production of organ specific antibodies as well as an accompanying thyroiditis (Witebsky and Rose 1956). An extract of the animals' own thyroid has been shown to have the same effect. It is of some interest to note that Witebsky who was a powerful champion of Ehrlich's "horror autotoxicus" notion should have been the first to describe this condition. The experimental auto-immune disease is characterised by a lymphoid invasion of the thyroid gland which has its histological parallel in human thyroid disease in which auto-antibodies have been described (Roitt et al. 1956). It is postulated that the destruction of the gland is brought about by a progressive chain reaction between the antibodies and the thyroid antigens against which they are directed (Doniach and Roitt 1959). The circulating antibodies found in this disorder have been shown to be gamma globulins by Korngold et al. (1959) and the cytotoxic effect of serum from these patients on thyroid cells in tissue culture (Pulvertaft 1959, Irvine 1959) has been shown to be present in the gamma globulin fraction by the present writer (see Irvine 1962).

An example where the experimental model provides evidence which strongly supports an auto-immune basis for the disease, but where the connection with human disease is not proven, is the group of demyelinating diseases which includes multiple sclerosis, post infectious disseminated

encephalomyelitis and the paralytic disease following small-pox and rabies vaccination. The relationship of paralysis to rabies vaccination using the traditional Pasteur method of immunisation with infected rabbit spinal cord attenuated by drying, had been appreciated for many years. The possible relationship of nerve tissue components of rabies vaccine to the incidence of this paralytic disease was noted by Koritschoner and Schweinburg (1925) who refer to post innoculation paralysis observed as early as 1888 by Gonzales in Barcelona. They report that when Pasteur was told of this, he explained the findings as due to what he called "false rabies", i.e. partly attributable to the vaccine having half-cured a case of paralytic rabies, and partly due to hysteria. These authors,after describing 39 cases of post innoculation paralysis, give details of experiments in which 78 rabbits were innoculated with rabbit spinal cords, and 15 of these developed paralysis of the limbs. These observations were confirmed by Rivers et al. (1933), and an associated demyelination noted. The use of Freund's complete adjuvant containing tubercle bacilli was found to enhance the experimental disease in animals (Kabat et al. 1947). Circulating antibodies to nerve tissue did not appear in all the animals in which the disease occurred, nor was their presence correlated with the severity of the process (Raffel 1961). Although serum from a paralytic animal transferred to a normal animal would not induce the disease, transferred lymphoid cells did do so (Paterson 1959), and

delayed hypersensitivity reactivity is now favoured as the probable basis of the experimental disease. An important connection between the experimental model and human demyelinating disease is the fact that anti-brain antibodies have been found in the sera of some patients with multiple sclerosis as well as in the post rabies vaccine disease and in the experimentally induced syndrome. Positive skin tests to brain substance, on the other hand, have not been demonstrable in the human diseases while they can be elicited in some species of experimental animals (Raffel 1961).

There are many other diseases for which an auto-immune basis has been suggested in which the evidence is slender and controversial. An example of such a disease is glomerulonephritis. The report of Mellors and Ortega (1956) that gamma globulin was localised by the fluorescent antibody technique in the glomeruli of kidney sections of patients with this disease is criticised by Cruickshank (1958,1959) who reports carefully controlled studies in which this work was not substantiated. He failed to find any evidence of an increase in the globulin content of the eluates from glomeruli of glomerulonephritis kidneys over the globulin content of eluates from normal glomeruli. He also obtained negative results using precipitation and complement fixation, when sera and eluates were tested with extracts of kidney tissue. Cruickshank points out that as circulating heterologous antibodies to kidney had been found to be rapidly bound to the tissues, auto-antibodies therefore might be more likely to be bound to the tissues than be found free in the

circulation. However, tissue localisation studies using the fluorescent antibody method were found to be consistently negative. Finally, he points out that there is neither evidence of excessive activity in the reticuloendothelial system nor plasma cell infiltration of the kidney in patients with nephritis.

The connective tissue diseases, and in particular the condition of systemic lupus erythematosus, have provided a fruitful field for the study of auto-immune phenomena.

Systemic lupus erythematosus was first described by Kaposi in 1872. He observed patients whose illness was characterised by fever, toxic manifestations, and cutaneous lesions resembling those of erysipelas to which he gave the name "erysipelas perstans faceii". The significance of the systemic manifestations of the disease, and the relationship of the visceral lesions to those in the dermis was first emphasised by Osler in 1895 who recognised that severe attacks of the disease could occur without cutaneous manifestations. Osler's remarkable description of the disease made without the benefit of pathological studies included the following: "By exudative erythema is understood a disease of unknown aetiology with polymorphic skin lesions - hyperaemia, oedema, and haemorrhage - arthritis occasionally, and a variable number of visceral manifestations of which the most important are gastro-intestinal crisis, endocarditis, pericarditis, acute nephritis and haemorrhage from the mucous surfaces". In addition, he recorded

splenomegaly in 2 of the 11 cases on which his description was based.

Libman and Sacks described non-bacterial endocarditis associated with skin lesions in 1924, and the cardiac lesions were described in detail by Gross between 1932 and 1940, including a description of haematoxylin staining bodies in the lesions. Detailed reports of other pathological changes were given by Klemperer et al. (1942) who described the essentially degenerative disturbance affecting the collagen of the tissues as "fibrinoid degeneration". He found haematoxylin bodies in 41 of 45 cases of the disease widely distributed throughout the body in the heart, kidneys, lymph nodes and synovia. He considered that these bodies were due to an alteration in the nuclei of mesenchymal cells, involving partial depolymerisation of D.N.A. by disturbed enzyme activity. The possibility that this change was brought about by a serum factor which released intra-cellular D.N.A'ase from inhibition, thus allowing it to depolymerise the nuclear D.N.A. was suggested by Kurnick et al. (1953).

Since the first description of the disease there has been much speculation about the aetiology of the condition. Suggestions have been made that the disease had an infective origin and tubercular, staphylococcal, streptococcal and viral infections have been all proposed but not substantiated (see Harvey et al. 1954). Endocrine factors have also been proposed in view of the frequency which the disease affects females in the reproductive phase (see Harvey et al.op.cit.) The work of Rich and Gregory (1947) showing that of 45 rabbits subjected

to protracted anaphylactic reactions of the serum sickness type 18 animals developed lesions in the branches of the coronary arteries comparable to those seen in Systemic Lupus, resulted in a suggested allergic or hypersensitivity cause of the disease, and this has been linked with the widespread use of chemotherapeutic agents which induce sensitivity reactions. A wide range of what are considered to be auto-immune phenomena have been described in Systemic Lupus including antibodies to red blood cells, thrombocytes, white blood cells and, more recently, to subcellular components.

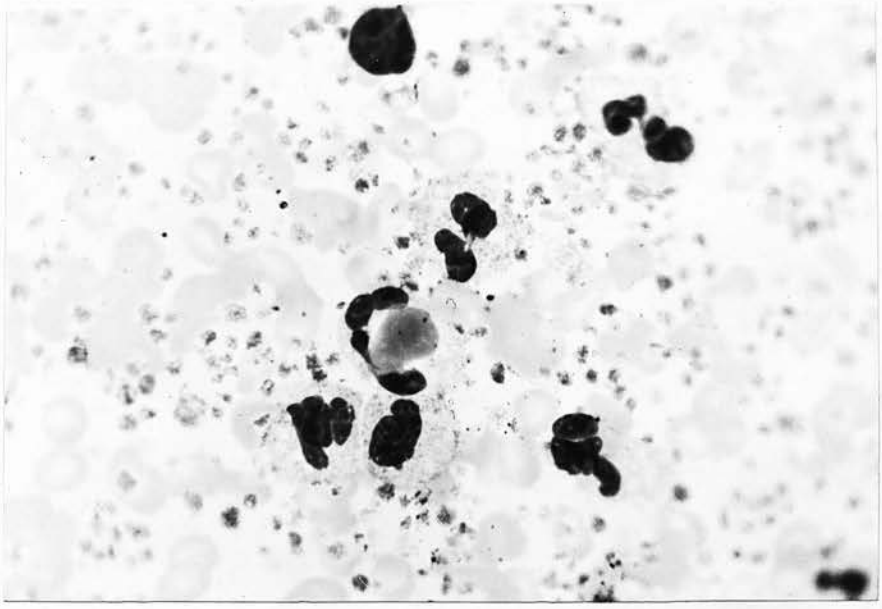
Haemolytic anaemia has long been recognised as a common feature of the disease. Figures ranging from 3% to 75% incidence have been reported, and many of these cases were shown to have a positive Coombs test (Zoutendyk and Gear (1950) Shearn and Pizofsky (1952) Dubois (1952) Harvey et al.(1954). The responsible factor has been isolated in the gamma globulin fraction of the serum (Fudenberg and Kunkel 1957). Multiple post transfusion antibodies were found frequently (Kuhns and Bauerlein 1953; Callender et al. 1945; Michael et al. 1951), and agglutination and clumping of red cells in conjunctival vessels was seen occasionally (Harvey et al. op.cit.).

Abnormalities of blood coagulation have been frequently reported in the disease. Osler (1895) noted that purpura was a frequent finding in his cases. Figures ranging from 6% to 52% incidence of thrombocytopenia have been reported in numerous studies (see Harvey et al.op.cit. 1954).

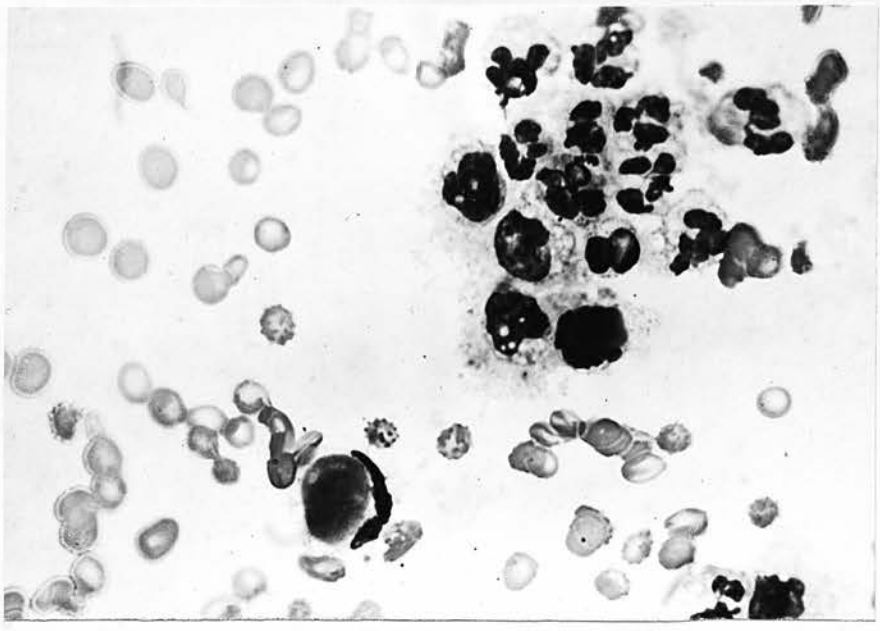
Seligmann (1958) reported the finding of anti-thrombocytic antibodies in more than half of 19 cases examined, and Loeliger (1960)

Plate 1.a

Lupus erythematosus cell (L.E. cell)



x 900

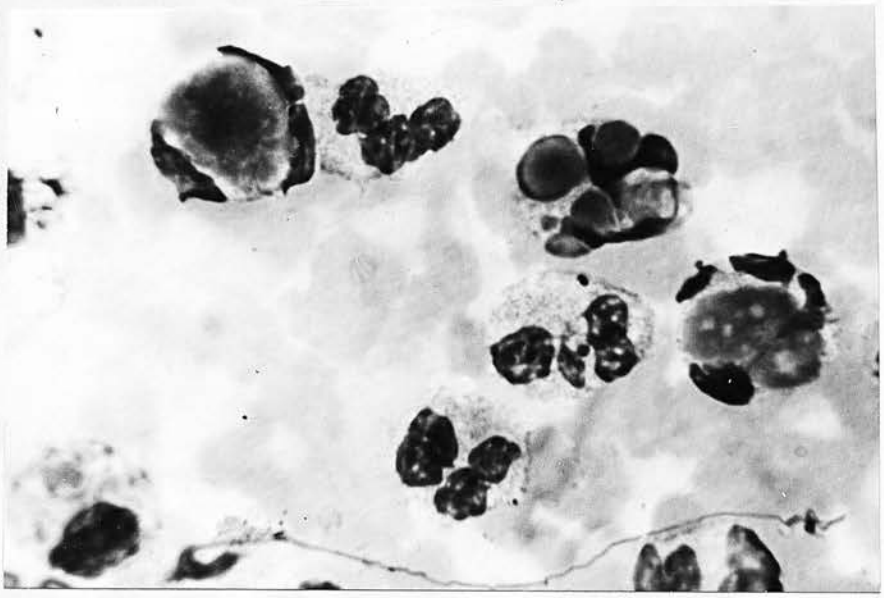


x 650

Note pale staining homogeneous inclusion surrounded by polymorph cytoplasm. (Wrights stain)

Plate 1 b.

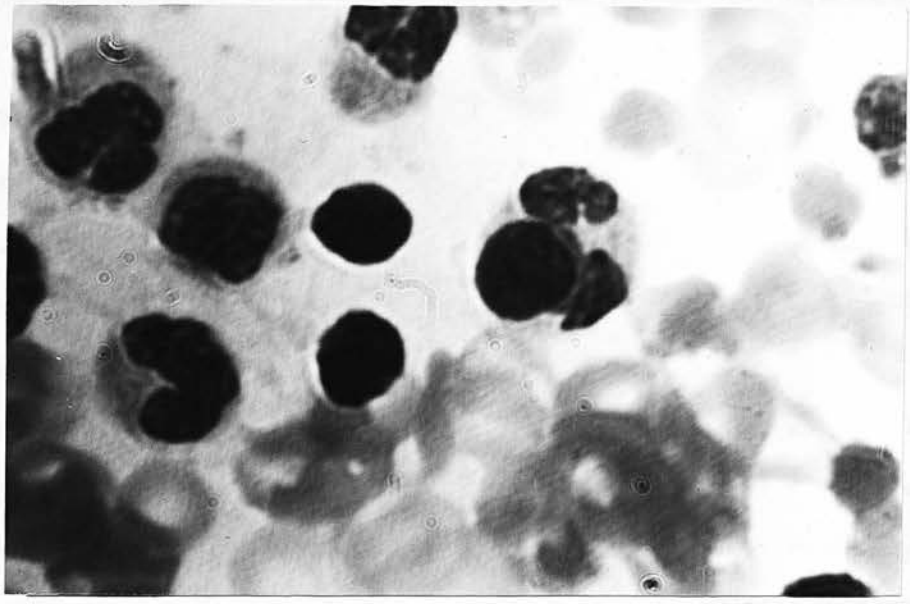
L.E. cell



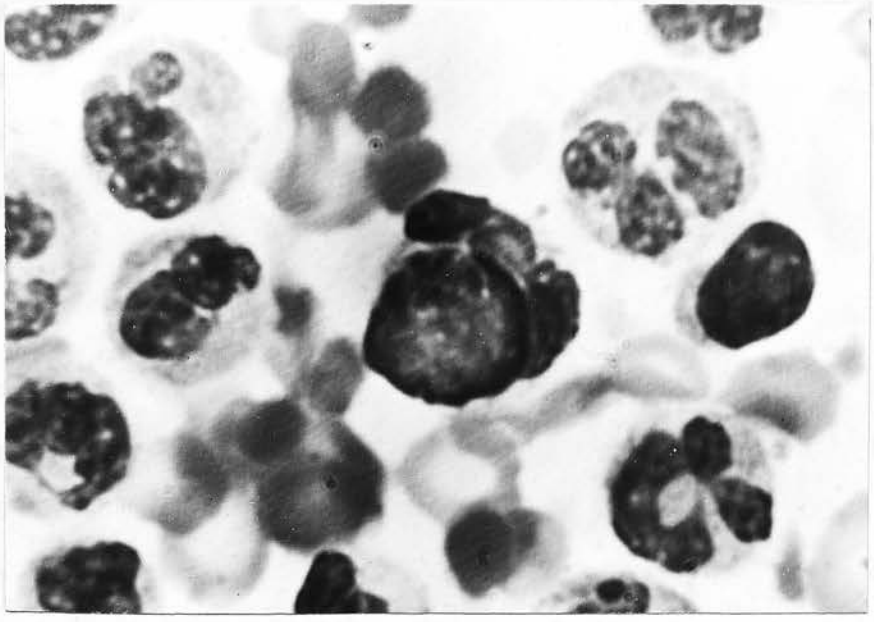
x 2000

Note multiple pale staining inclusions, some showing marked nucleolysis. (Wrights Stain)

Plate 2. Nucleophagocytosis.



x 2000



x 2000

Note dark staining inclusion (lymphocyte nucleus) with well marked chromatin pattern. (Wrights stain)

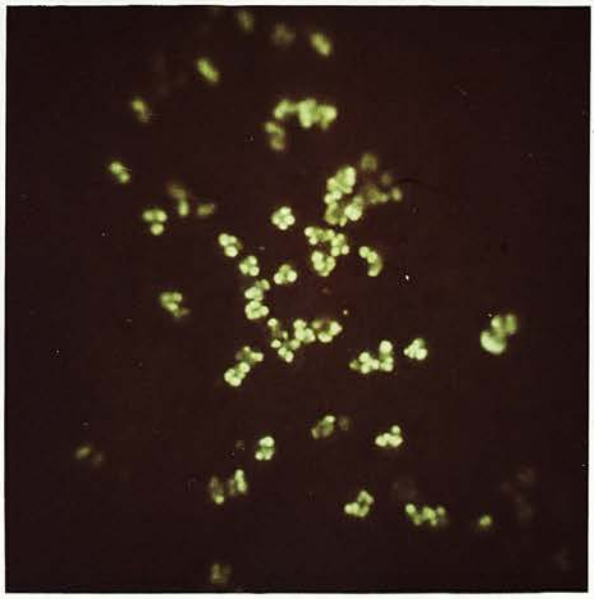
described a serum gamma globulin factor which was active against thrombocyte factor 3, intrinsic thromboplastin and tissue thromboplastin. Other descriptions of abnormalities at the thromboplastin stage are reported by Lee and Saunders (1955) and Frick (1955), and at the stage of conversion of prothrombin by Conley and Hartmann (1952), and Bonnin et al. (1956). Anti-cytoplasmic complement fixing antibodies are described in the sera of some patients by Mackay and Gajdusek (1958), Asherson (1959) and Deicher et al. (1960). These antibodies are reported to react with the subcellular components, mitochondria and microsomes and with soluble cellular protein. An incidence of 25% to 58% of leucopenia has been reported in various studies (see Harvey et al. 1954). Anti-leucocyte antibodies have been reported to occur spontaneously, Seligmann (1957) Dausset et al. 1961), and following transfusion (Zimmerman et al. 1955).

The L.E. cell phenomena was first described by Hargraves, Richmond and Morton in 1948 in the bone marrow of patients with systemic lupus erythematosus, and later demonstrated in the blood of these patients (Sundberg and Lick 1949; Gonyea et al. 1950; Moffat et al. 1950). The phenomenon has been shown to take place in two stages: the alteration of the nuclei of dead leucocytes, usually polymorphs, to form a homogeneous, pale, basophilic, Feulgen positive mass: and the phagocytosis of this material by an intact polymorph to form an L.E. cell (Plate 1a.1b)(Rohn & Bond 1952). The phenomenon can be demonstrated using the serum of a patient and the cells of a normal individual (Moffattet al. 1950). The specificity of the reaction for

systemic lupus erythematosus was considered established (Madden 1950, Haserick 1951, Lee et al. 1951, Dubois 1953, Stich et al. 1952) but has since been reported in a proportion of rheumatoid arthritis cases (Kievits et al. 1956, Ogryzlo 1957, See Marmont 1959), drug sensitivity (Delacretaz et al. 1954, Dustan et al. 1954, Perry and Schroeder 1954), and rare cases of liver disease (Mackay and Gajdusek 1958) and scleroderma (Marmont 1959). The serum factor responsible for initiating the phenomenon was identified in the gamma globulin fraction of the serum (Haserick 1950, Lee et al. 1951, Willkens et al. 1958, Fallet et al. 1958, Carlson and Mollerberg 1958, Goodman et al. 1959,1960, Scheidegger et al. 1960). There are a number of disadvantages which detract from the value of the L.E. cell test as a routine diagnostic procedure. Variation in the sensitivity of the test has been shown by the wide range in the incidence of positive tests in rheumatoid arthritis reported by different workers. (Harvey et al. 1954, Wilkinson and Sacker 1957, Kievits et al. 1956, Ogryzlo and Smythe 1957, Friedman et al. 1957). The test is time-consuming and is dependent on distinguishing between fine differences of morphology in order to decide between true L.E. cells and so-called Tart cells and artefacts (Plate 2). The most common difficulty encountered is the finding of phagocytosed nuclear material which still shows some chromatin pattern and retains most of the basophilia of a normal nucleus. Artefacts of this type have been noted, particularly in preparations made from the blood of patients with rheumatoid arthritis (Delacretaz et al. 1954, Marmont 1959).

Plate 3. Fluorescence photomicrograph.

Demonstration of anti-nuclear factor on leucocyte nuclei (Coon's indirect technique)

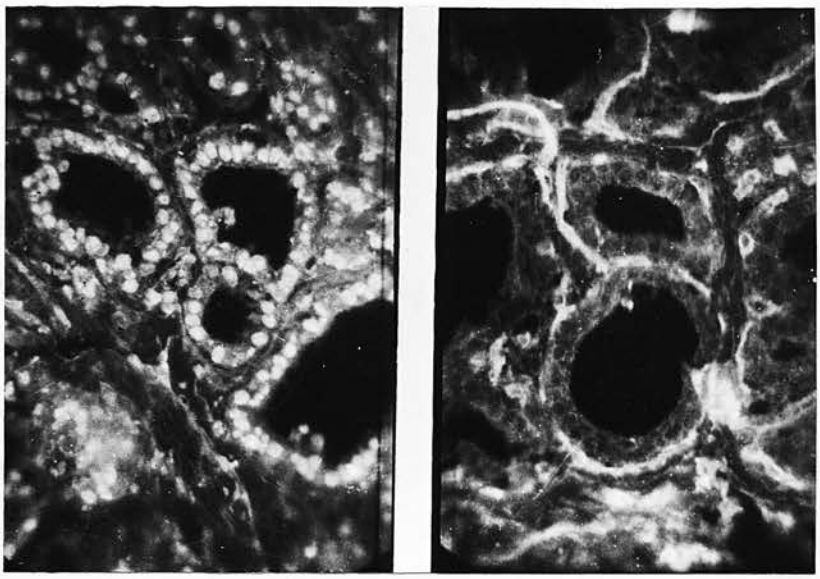


x 400

Note bright apple-green nuclei against dull green cytoplasm.

Plate 4. Fluorescence photomicrograph.

Positive and negative anti-nuclear factor test on infant thyroid sections.



positive negative

x 600

Note the bright staining "beads" of the acinar nuclei in the positive perparation, unstained "holes" in the negative preparation. (Bright strands of staining in negative preparation due to blood group antibody in test serum).

It was the author's task in the Spring of 1957 to carry out routine testing for the presence of L.E. cells at the Rheumatism Unit, Taplow, and the difficulties mentioned above were encountered often enough to create a practical problem. It was decided to investigate the possibility of using a fluorescein labelling technique to detect the L.E. serum factor. As this had been shown to be a globulin (Haserick 1950) it was hoped to demonstrate the coating of this globulin on the cell membrane of white cells since it was thought that breakdown of the cell membrane might be required as a preliminary step to allow the release of D.N.A'ase from inhibition (Kurnick's hypothesis that D.N.A'ase inhibitor was released by an L.E. serum factor). When the problem was taken to Dr. E.J. Holborow and Mr. G.D. Johnson who were using the fluorescence microscope to study bloodgroup substances in gastric mucosa, it was decided to apply the indirect method of Coons and Kaplan (1950), which visualises the uptake of human globulin by means of fluorescein conjugated anti-human globulin, to polymorph smears. When leucocyte films were treated with L.E. cell positive serum and then stained with fluorescein conjugated anti-human globulin serum, it was found that the leucocyte nuclei fluoresced brightly when examined microscopically by ultra-violet light (Plate 3). This nuclear staining seemed to be even more significant than the expected cytoplasmic staining would have been in view of the fact that the characteristic fibrinoid material, haematoxylin bodies and the L.E. cell inclusion all contain altered nuclear material (Klemperer et al.)(op.cit). The next few weeks were

spent on trying to standardise the technique, which at that time was giving patchy staining on the white cell films and a fair amount of background staining due to serum proteins from the blood used for the smears. At this point, Professor E.G.L. Bywaters returned from the 9th International Congress of Rheumatic Diseases and reported the findings of G.J. Friou. In a search for antibodies with an affinity for specific cell structures in patients with diffuse connective tissue disease, Friou had found and reported to the Congress that L.E. cell positive serum reacted with the nuclei of connective tissue, endothelial cells, blood vessels, serous surfaces, muscle, and with normal tissues. He regarded the nuclear antigen as likely to be desoxyribonucleoprotein or closely bound to it. This information led the author to use the indirect Coons fluorescent technique on frozen tissue sections cut in a Cryostat, and staining of the cell nuclei of kidney, spleen, skin, heart muscle and thyroid tissue demonstrated with an L.E. cell positive serum. Normal serum did not give nuclear staining (Plate 4). The thyroid was selected as giving the best results - because of the regular and predictable arrangement of the cell nuclei - and the findings were confirmed with 2 sera from patients with systemic lupus and one from a patient with rheumatoid arthritis and a positive L.E. cell test (Holborow, Weir and Johnson 1957). In this communication the term "anti-nuclear factor" was first used. Publication of these findings coincided with a report from the Rockefeller Institute by Holman and Kunkel (1957) concerning the factors.

affecting L.E. Cell formation which described the fluorescent antibody localisation of gamma globulin on L.E. cell inclusions. A short time later, P. Miescher sent the author a copy of his study reported in Experientia in 1954 (Miescher and Fauconnet 1954) showing that L.E. cell forming activity could be absorbed from a serum by the addition of isolated cell nuclei. The accumulating evidence posed the problem:- was the anti-nuclear factor an antibody, were the nuclear materials behaving as antigens, and was the phenomenon an example of auto-immunity? In addition, about this time, the author demonstrated the anti-nuclear factor in a patient with rheumatoid arthritis with a negative L.E. cell test. The further question of specificity of the anti-nuclear staining and its relation to the L.E. cell factor arose. It was in an attempt to answer some of these questions that the author undertook a study between June 1957 and August 1960 at the M.R.C. Rheumatism Research Unit, Taplow, supported by the Empire Rheumatism Council. The present work describes the result of an investigation into the incidence of anti-nuclear factor in nearly 1000 human sera, and an immunological study of the problems indicated above. Clinical information was ascertained, partly by reference to the case notes of the patients, and partly in consultation with the medical staff in charge of the patients. It is presented not exhaustively but in-as-much as it appeared to be relevant to these immunological problems.

CHAPTER 1.

MATERIALS AND METHODS

Clinical material

The sera used in this investigation were obtained with the co-operation of the clinical and laboratory staff of the units detailed below. The specimens, kept in screw-cap containers, were stored at -20°C. in a deep freeze cabinet for periods up to three years. No preservative was added and no deterioration of anti-nuclear factor acitivity was found in specimens kept up to three years. Samples of serum were removed with a clean pipette without sterile precautions and tested for anti-nuclear factor activity in batches of 20-30 specimens.

Systemic Lupus Erythematosus: Rheumatism Unit, Hammersmith Hospital.

Rheumatism Unit, Taplow
(Professor E.G.L. Bywaters)

The Middlesex Hospital
(Dr. D. Doniach)

The University Hospital, Leyden
(Dr. W. Hijmans)

The criteria used for the selection of cases of this disease were those submitted by Bywaters and Ansell to the M.R.C. Collagen Panel in 1952. (Appendix 1).

Rheumatoid Arthritis: Rheumatism Unit, Hammersmith Hospital.

Rheumatism Unit, Taplow.

This group were selected using the American Rheumatism Association criteria 1959 (Appendix 2).

Discoid Lupus: St. Bartholemews Hospital Skin Department (Drs. Scott and Rees)

Thyroid Disease: The Middlesex Hospital (Dr. D. Doniach and Dr I. Roitt)

These were cases of Hashimotos disease and the diagnosis had been confirmed by the presence of thyroid antibodies detected by the tanned cell agglutination test, by precipitation or by complement fixation.

Liver Disease: Hammersmith Hospital (Dr. S. Sherlock and Dr. Dölle)

These cases included a variety of liver diseases, see table 2.

Pulmonary Tuberculosis: Pinewood Hospital, Berks.

These were confirmed cases in the acute or convalescent stage.

Normal sera: These were obtained from laboratory staff, ante-natal patients and from normal individuals used as controls of a family survey of Rheumatoid disease (Dr. Ansell).

Technical procedures and preparation of materials

The following sections include descriptions of the preparation of materials and the main technical procedures used throughout the experimental work. Further techniques relevant to individual experiments are described in the appropriate sections.

Anti-nuclear factor test

The test makes use of the Coons and Kaplan (1950) fluorescent antibody technique. Antibody labelled with fluorescein isocyanate (or isothiocyanate) is used to visualise the homologous antigen in cells

of smears or tissue sections examined in a fluorescence microscope with a high pressure vapour light source. In this test the "indirect" method is used in which globulin uptake by tissue sections is demonstrated by treatment with anti-human globulin serum conjugated with fluorescein isocyanate or isothiocyanate. Sections pre-treated with serum containing anti-nuclear factor show bright overall apple-green fluorescence of the cell nuclei when viewed microscopically by ultra-violet light, whereas control sections pre-treated with normal serum show no fluorescent staining (Plate 4).

Preparation of tissue sections

The selection of cell nuclei in a suitable form for the test is an important consideration. Preparations of nuclei tested include smears of cell nuclei prepared from tissue homogenates, human leucocyte films and monolayers of cells grown in tissue culture. None of these preparations was ideal owing to the affinity of such preparations for serum proteins which subsequently reacted with the fluorescein conjugated antiserum and led to excessive background staining. This non-specific staining was most marked with preparations of nuclei freed from cytoplasmic material by physical means or by trypsinisation. Cell nuclei prepared in this way took up serum proteins very readily from any serum whether from normal individuals or from lupus patients. Experience with tissue culture monolayers indicated the need for some cell damage before the nuclei could take up the serum factor from lupus

sera. Monolayers treated directly with serum failed to take up the factor whilst preparations which were first frozen and thawed would take up the factor. The most satisfactory nuclear preparations for routine use were made from thyroid tissue because of the orderly and predictable arrangement of the cell nuclei. The cutting of the sections and thawing from the frozen state was sufficient to expose the cell nuclei so that they could take up the anti-nuclear factor. Human neonatal thyroid was preferred to adult tissue because the latter usually contains numerous autofluorescent granules in the epithelial cytoplasm, and much interstitial autofluorescent collagen, both of which interfere with observation of the sections.

Human neonatal thyroid from very fresh post-mortem specimens was cut into small blocks, instantly frozen in a sealed container in solid carbon dioxide and alcoholmixture (approximately - 70°C.) transferred to a freezing cabinet at -20°C. and stored without thawing. Sections were cut in a cryostat chamber on a microtome at 6μ., and mounted on slides and dried in air by exposure to an electric fan. Each section was covered with a few drops of test serum and incubated in a moist chamber at 37°C. for 30 minutes. After incubation, the serum was carefully washed off in two changes of buffered saline pH 7.0 ($Na_2HPO_4 12H_2O$, 2.14g. KH_2PO_4. 0.54g. and NaCl 8.70g. per litre) for 20 minutes, fixed in 90% alcohol for 5 minutes, washed again, and stained with a fluorescent conjugate prepared from anti-human globulin serum. After 30 minutes, the sections were again washed and mounted in buffered glycerine (one part of buffered saline to nine parts of glycerine pH 7.0) under a glass coverslip.

Preparation of conjugates

Rabbit and horse anti-human globulin sera were conjugated with fluorescein isocyanate or isothiocyanate according to the method of Coons and Kaplan (1950) and Marshall et al (1958) as follows:-

The globulin fraction of the antiserum was prepared by 50% saturation with ammonium sulphate in the cold for 2 hours. The precipitate was separated from the supernatant albumen by centrifugation at 4000 r.p.m. in an M.S.E. major refrigerated centrifuge at 4°C. for 45 minutes. After removal of the supernatant the precipitate was dissolved in a small quantity of distilled water, just sufficient to completely dissolve the precipitate, and dialysed overnight against N saline in the cold. The total protein of the dialysed globulin fraction was estimated by the quantitative Biuret method. The optimal quantity of fluorescein isocyanate for each mg. of protein was estimated by Coons and Kaplan as 0.05 mg. per mg. of protein. Above this level no further conjugation takes place and progressively larger amounts of protein are denatured. For conjugation, the final reaction mixture is made up to contain 10 mg. per ml. of protein and consists of 0.05 mg. fluorescein isocyanate per mg. of protein; 15% of dioxane and 7% acetone, with the final addition of 15% 0.5 M.carbonate - bicarbonate buffer pH9 and saline to 100%. This procedure was carried out in the cold with chilled reagents and glassware. The chilled protein was added to the mixture of solvents, buffer and saline. The isocyanate solution was added dropwise with mechanical stirring, and the stirring process continued in the cold for 18 hours. The mixture was then

dialysed against buffered saline pH7 (0.15M.NaCl containing 0.1M. phosphate) on a revolving stage, changing the buffer frequently until the final dialysate was colourless (2-3 days). The conjugate was then centrifuged in the cold at 4,500 r.p.m. in the M.S.E. major centrifuge for 45 minutes and stored at $4^{\circ}C$.

Where the fluorescein isothiocyanate compound was used, conjugates were prepared from the ammonium sulphate precipitated globulin fraction which was diluted to 10mg/ml. concentration with the 0.5M. carbonate - bicarbonate buffer pH9, so that the final mixture contained 10% buffer. The solution was cooled to $4^{\circ}C$. and 0.05 mg. of fluorescein isothiocyanate was added for each mg. of protein. The mixture was then transferred to a rotary shaker and agitated 12-18 hours in the cold. The conjugate was then treated in the same way as the isocyanate preparation.

Absorption of the conjugates

Some conjugates stained the tissue in a so-called non-specific manner resulting in a blue-green overall fluorescence of the whole tissue. This interfered with visualisation of the sites of specific uptake of antibody, which should ideally stand out bright apple-green fluorescent areas against a faint greenish background. Coons recommended that such conjugates be absorbed with organ powder. The powder used in this work was prepared from fresh rabbit liver which was homogenised with saline in a waring blender, repeatedly washed with acetone and finally dried

and powdered in a mortar and pestle. 100 mg. of the resulting fine powder was used for absorbing 1 ml. of conjugate. The carefully shaken mixture was left for one hour, after which the conjugate was recovered by centrifugation.

Mounting medium for sections

This consisted of buffered glycerol made up by using 10 parts of glycerol to one part buffered saline pH7.0 (0.15M. NaCl. containing 0.01M. phosphate).

The Fluorescence Microscope

The microscope used in this work was a Cooke Troughton and Simms Research Microscope fitted with a dark ground condenser and with x10 x 45 and x 95 achromatic and fluorite objectives. The light source consisted of a Mercury vapour lamp HB200 with choke and switch gear. A heat filter was mounted in front of the lamp together with an adjustable field lens for focusing the light on the substage mirror. An ultra-violet passing filter (Kodak Wratten 18B) was fitted in front of the field lens. Neither a quartz condenser, eyepiece filter or copper sulphate bath was required in this work as the dark ground condenser was used throughout.

Preparation of antisera

The anti-globulin sera used in this study were either prepared in the laboratory (1 and 2) or obtained commercially (3 and 4).

(1) Cold precipitating globulins were prepared from human sera by the method of Svartz and Schlossman (1953). Serum was inactivated by heating at 56°C. for 30 minutes and diluted 1/2 with physiological saline. Fourteen volumes of ice-cold sterile distilled water were added and the mixture was left at 4°C. for 48 hours. The precipitated material was re-dissolved in saline and used as the immunising antigen. Rabbits were given subcutaneous injections of 1 ml. of the cold globulin solution (containing 20 mg. protein) emulsified with Freunds complete adjuvant. The injection was repeated three times at weekly intervals and then after an interval of four weeks a booster dose of a further 1 ml. of the globulin solution with adjuvant was given. Another similar injection was given four weeks later. One week after the last injection, the animals were bled and the serum collected. The results of immunoelectrophoresis using this serum (conjugated with fluorescein) are shown on plate 5.

(2) An anti-human globulin serum was prepared by the method of Milgrom et al (1956). One ml. of packed rabbit red cells were mixed with 10 ml. of inactivated (56°C. for 30 minutes) pooled human serum and left at room temperature for 30 minutes with occasional shaking. The red cells were then washed three times with saline, re-suspended

in 3 ml. of saline and injected into the marginal ear vein of a rabbit. The injection was repeated twice weekly for six weeks and the antisera collected 10 days after the final injection.

(3) Horse anti-human serum - supplied by the Institute Pasteur.

(4) Coomb's anti-human globulin serum prepared in rabbits immunised with human globulin (Burroughs Wellcome).

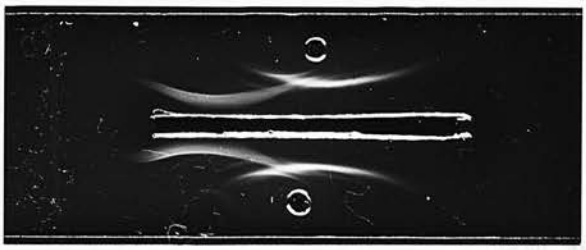
These antisera when conjugated with fluorescein as described were all able to detect the uptake of anti-nuclear factor. However, they showed varying degrees of non-specific background staining, and almost all of them required absorption with dried organ powder. The most suitable serum was that prepared against human cold globulins which required no absorption with organ powder, and the serum which gave the most background staining was the conjugate of Coomb's serum. Plate 5 shows the immunoelectrophoretic pattern obtained with these two conjugated antisera against whole human serum. The anti-human globulin conjugate which proved most useful showed only one major component against the gamma globulin component of the serum, whilst the other antiserum showed precipitin bands against a large number of serum components. The latter antiserum therefore would be likely to detect the uptake of a variety of serum proteins on tissue sections and result in the background staining encountered.

Plate 5.

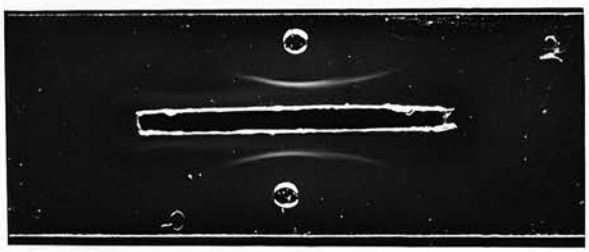
Immunoelectrophoretic patterns with "anti-human globulin" conjugates (Immunoelectrophoresis in 1% agar in veronal buffer pH 8.6. Electrophoresis for 4 hours at 1 milliamp per cm. in veronal buffer pH 8.6. Immunodiffusion 24 hours at 37°C.)

+

Coombs "anti-human globulin" conjugate No. 4



"Anti-human globulin conjugate"No. 1.



Note multiple precipitin bands against many serum components with conjugate No. 4, compared with one main band against the gamma globulin component with conjugate No. 1.

Technique for L.E. cell test

(a) Where fresh blood was available a modification of the clot method of Zimmer and Hargreaves (1952) was used as follows:-

Clotted blood was incubated at 37°C. as soon as possible after having been taken from the patient. After two hours the container was shaken (in order to damage some of the leucocytes and make them more susceptible to L.E. cell formation) and re-incubated for a further 20 minutes. At the end of this period, the mixture of serum and cells was separated from the clot, using a Pasteur pipette, and centrifuged in a Wintrobe tube at 2,500 r.p.m. for 5 minutes. The buffy coat containing the leucocytes was carefully removed and films prepared on clean glass slides. The films were stained with Wright's stain and examined under the low power objective. The high power was used for selected areas of high concentration of polymorphs and the oil immersion was used only for detailed examination of a possible L.E. cell. A five minute examination of each preparation was carried out.

(b) Where serum only was available the indirect technique of Schultz et al (1955) was used as follows:-

One or two drops of fresh human blood obtained by venepuncture (or finger-prick when only a few preparations were required) were placed on the centre of a clean glass slide and allowed to clot in a damp chamber at 37°C. After 20 minutes the clot was washed off gently with a stream of warm phosphate buffered saline (pH.7.0). This left the leucocytes (which had migrated to the periphery of the clot) on the

surface of the slide as a visible film. This film of living cells was kept damp and immediately layered with a few drops of test serum. A coverslip was placed over the preparation and it was re-incubated at 37°C. for 1 hour in a damp chamber. The coverslip was then removed and the excess serum allowed to drain off the slide by placing the slide on its side in a box containing a layer of blotting paper. After the slide had dried, it was stained with Wright's stain and examined as above for L.E. cells.

Nucleoprotein Preparation

Calf thymus nucleoprotein was prepared by the method of Mirsky and Pollister (1946). Fresh tissue, frozen at - 70°C. immediately after death was homogenised in a waring blender with 0.14 M. saline at 4°C. After repeated homogenisation and centrifugation (2,500 r.p.m. in a M.S.E. Major refrigerated centrifuge), until the supernatant was no longer cloudy, the deposit was dissolved in IM. saline, reprecipitated three times by the addition of six volumes of water, redissolved in IM. saline and stored at 4°C. The calf thymus nucleoprotein obtained in this way contained 1.2mg/ml. of DNA as measured by ultra-violet absorption in a Unicam spectrophotometer(Fig. I) and 1.2mg/ml. of protein as measured by the Biuret method as follows:-

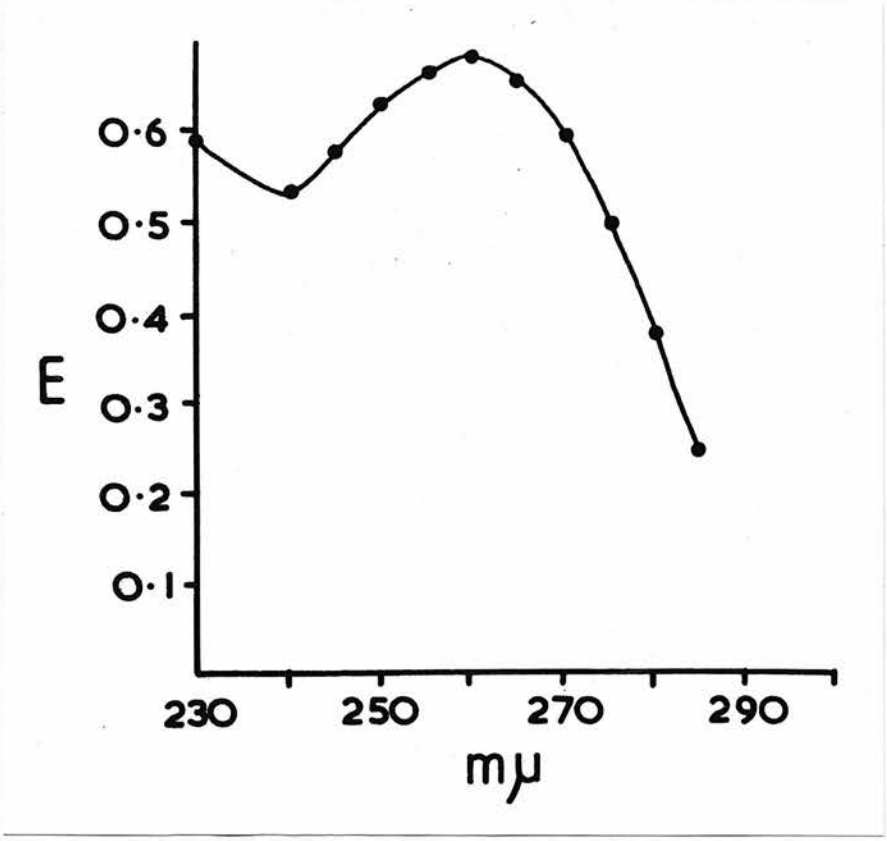
4.8 ml. of Biuret reagent and 0.2 ml. of test solution were mixed, left to stand at room temperature for 30 minutes, and the colour compared with a control containing water instead of the test solution in a Spekka

Figure 1.

D.N.A. Content of Calf Thymus

Nucleoprotein

Absorption of nucleoprotein preparation at 230 to 285 mμ.



Note peak at 260 mμ.

spectrophotometer. The protein concentration was then obtained from a standard curve. The same method of preparing nucleoprotein was used for obtaining guinea-pig liver nucleoprotein. This latter preparation required to be re-precipitated six times by six volumes of water from IM saline in order to remove the excess protein material. The final concentration was 1.2mg/ml. of DNA and 1.2mg/ml. protein.

Calculation of D.N.A. concentration in calf thymus nucleoprotein

Given that a 0.05% solution of sodium D.N.A. gives an extinction of 9.8 at 260 mμ. (Davidson 1957).

Thus an extinction of 0.68 will be given by a 0.0035% solution of DNA (0.68/9.8 x 0.05).

As extinctions measured with a solution of nucleoprotein diluted 1/50 the concentration of the undiluted nucleoprotein preparation is 50 x 0.0035% = 0.175%. Calculated nucleoprotein concentration 1.75 mg. D.N.A. per ml.

Feulgen staining procedure

The preparation was fixed in 10% neutral formaldehyde for 5 minutes and then washed in tap water for 5 minutes. Acid hydrolysis was then carried out by immersing the preparation in N.HCl for 10 minutes at 60°C. Following this it was transferred to cold Schiffs reagent (Lilly) for 15 minutes and rinsed in three changes of SO_2 water. The preparation was then washed in tap water for 5-10 minutes and taken through 70% and 90% alcohol into 0.01 Fast Green in 95% alcohol for 30 seconds. The preparation was finally rinsed in 95% alcohol and xylene, and mounted.

Electrophoresis and immunoelectrophoresis

Electrophoresis was carried out in the Shandon Universal Electrophoresis Apparatus. Electric current was supplied by a Vokam power pack which provided an adjustable voltage with a constant current at each selected voltage. The current was passed into platinum wire electrodes which lay one on either side of a central partition in the floor of the perspex electrophoresis box. Veronal buffer pH 8.6 was introduced into either side of the partition to fill both chambers to an equal depth (about 1 cm.) The buffer consisted of:- Veronal (Diethylbarbituric acid) 1.38 gms. per litre, Sodium veronal 8.76 gms. per litre, Calcium lactate 0.38 gms. per litre, made up in distilled water. Cellulose acetate paper (Oxoid) was cut into strips of the required length (usually 10 cm.) and immersed in the buffer for 10 minutes. The paper was then blotted and placed so that it straddled the central partition and rested on a movable supporting bar in each chamber. Filter paper wicks extending from the ends of the paper into the buffer were held in position by means of pieces of perspex fitting over the supporting bars. The material to be electrophoresed was run on to the paper from a capillary tube, allowed to soak into the paper, and the current switched on to give a voltage of about 150 volts (usually requiring 1 to 2 milliamps for each electrophoretic strip). After 2 hours, the paper was removed with forceps and dried in an oven at 100°C. for 15 minutes. It was then stained with the protein stain Ponceau S (Gurr) 0.15% in 3% trichloracetic acid

for ten minutes and the excess stain washed out with 5% acetic acid. This stained the protein a bright red colour. An alternative stain which was used alone or following Ponceau S was Nigrosine (Gurr) 0.0025% in 2% acetic acid. Nigrosine stained the protein black over a period of a few hours and this gave good definition of protein patterns. Excess Nigrosine stain was removed with running tap water, and finally the paper strip was dried between two sheets of blotting paper.

Immunoelectrophoresis was carried out in the same apparatus, and glass microscope slides, covered with a layer of 1% agar dissolved in the veronal buffer, took the place of the electrophoretic strips. A pattern, consisting of a central longtitudinal trough 1 mm. wide and a small well on either side of this, had been cut into the agar on the slide. A sample of the material to be electrophoresed was placed in each of the small wells in the agar and electrophoresis was carried out at approximately 50 volts (requiring about 2 milliamps per slide). After 4 hours the slide was removed from the electrophoresis box, and a quantity of the anti-serum was pipetted in to the central trough. The preparation was placed in a damp chamber at 37°C. and diffusion allowed to take place for 16 hours, after which the preparations were examined for precipitin lines.

CHAPTER 2.

The anti-nuclear factor in human disease

The anti-nuclear factor test was carried out as described, using sections of infant thyroid cut in the cryostat. As nearly one thousand sera were tested over a considerable period, several different fluorescein conjugated "anti-human globulin" sera were used. The different conjugates, as already described in Chapter 1, varied in their ability to detect traces of serum protein adherent to tissue sections. The reason for this was discovered subsequently when it was found that anti-human globulin conjugate No. 1 gave only one main precipitin band with human serum in immunoelectrophoresis (plate 5). With this conjugate, the test sera could be used undiluted without showing background staining sufficient to interfere with the contrast of the stained nuclei against the surrounding tissue. The other conjugates which contained precipitating antibody against other serum components in addition to gamma globulin (plate 5) could only be used with test serum diluted to 1/10. At this dilution, the background staining was sufficiently reduced to be able to distinguish the specific nuclear staining when it occurred. Each series of tests included a known positive serum and a negative serum control. A control was carried out to show that the anti-human globulin had not altered in specificity after conjugation. This was confirmed also by the immunoelectrophoresis experiments (plate 5).

TABLE 1

Distribution of positive anti-nuclear factor and L.E. cell tests in disease groups and controls

Diagnosis	Anti-nuclear Factor			L.E. Cells		
	No. tested	Positive		No. tested	Positive	
		No.	%		No.	%
S.L.E.	63	62	98	63	58	92
Discoid Lupus Erythematosus	75	10	13	75	0	0
Rheumatoid Arthritis	132	19	14	95	3	3
Stills Disease	100	13	13	60	1	2
Thyroid Disease	110	14	13	107	0	0
Liver Disease	39	5	13	39	1	3
Scleroderma	8	0		8	0	
Dermatomyositis	4	2		4	0	
Rheumatic Fever	56	0				
Normal sera	133	2 *	1.5			
Miscellaneous hospital cases	60	1 *	1.7			
Pulmonary Tuberculosis	65	2 *	3.00			
Totals :	845	130		451	63	

* L.E. cell negative.

For this, sections were layered with an anti-nuclear factor positive serum, followed by unconjugated anti-human globulin serum, and then with the fluorescein conjugate. The specificity of the conjugates was confirmed with each conjugate by the absence of nuclear staining under these conditions because the unconjugated antiglobulin had already combined with the anti-nuclear factor. Another control carried out was the demonstration that no nuclear staining occurred with anti-nuclear factor positive sera when the serum-treated sections were stained with a conjugate of different specificity. The particular conjugates used were human anti-A serum and goat anti-rabbit serum.

Systemic lupus erythematosus

Sixty-three cases of this disease, which fitted the diagnostic criteria used for the selection of the cases, were tested for the presence of anti-nuclear factor in their sera. This group included five cases which had been shown to have a negative L.E. cell test but which fitted the diagnostic criteria and could thus be included in the series. Table shows the results of anti-nuclear and L.E. cell tests on these sera. All 58 cases of systemic lupus with positive L.E. cell tests showed positive anti-nuclear factor staining at first testinf. Four of the five L.E. cell negative cases were positive for anti-nuclear factor, and one remained negative despite repeated testing over a period of three years. This case was a 21 year old woman who had presented three years prior to the commencement of this investigation a typical butterfly rash, loss of weight, pyrexia, loss of hair,

generalised lymphadenopathy, splenomegaly, arthralgia and leucopenia (below 5,000 w.b.c. per cu.mm.). At the time testing for anti-nuclear factor commenced, the E.S.R. had returned to normal and she appeared to have recovered following steroid therapy. Repeated tests for three subsequent years for anti-nuclear factor were negative and tests for L.E. cells were negative throughout the 6 year period.

Rheumatoid arthritis

In the rheumatoid arthritis group of 132 cases, 19 gave a positive anti-nuclear factor test; two of these were L.E. cell positive.* A third L.E. cell positive case was encountered which was persistently negative for anti-nuclear factor (14 tests during 2 years). This was a 45 year old woman with generalised active rheumatoid arthritis of 7 years duration. In the group of Stills disease, 13 of the 100 cases tested had a positive anti-nuclear factor test, and only one of the thirteen had a positive L.E. cell test.

Thyroid disease

This group consisted of 110 cases of Hashimoto's disease, including 14 cases complicated by thyrotoxicosis. Anti-nuclear factor was found in 14 of the 110 cases (2 of these positives were in the group with thyrotoxicosis). The L.E. cell test was uniformly negative.

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* In the preamble to the revised criteria for the diagnosis of rheumatoid arthritis, it is recommended that cases with a positive L.E. cell test, which had previously been completely excluded from the category of rheumatoid arthritis, now be considered together with rheumatoid arthritis patients, if they satisfy the criteria in every other respect. (See Appendix)

Other diseases

Ten of seventy-five cases of discoid lupus erythematosus showed anti-nuclear factor activity but were negative in the L.E. cell test. The liver disease group included 9 cases of cirrhosis which were considered to have followed infective hepatitis, and 4 out of the 5 positive tests occurred in the group (table 2). One of these 4 gave a positive L.E. cell test. Two of the 4 dermatomyositis cases tested had anti-nuclear factor, but the eight scleroderma cases and the 56 rheumatic fever cases tested were negative. The control sera tested fell into three categories:-

1) One hundred and thirty-three laboratory staff, ante-natal patients, and normal individuals used as controls of a family survey of rheumatoid disease. Two of the family survey control group were positive.

2) Sixty miscellaneous hospital cases, including chronic bronchitis, pneumonia, congestive cardiac failure, asthma, iron deficiency anaemia and Pagets disease. One of these cases was found to be positive. This was a case of Pagets disease with congestive cardiac failure which was negative in the L.E. cell test. Three other cases of Pagets disease were negative in the anti-nuclear factor test.

3) Sixty-five uncomplicated cases of pulmonary tuberculosis which were selected in an effort to have a homogeneous disease group unrelated to the 'connective tissue disease' group. Two of these cases were positive in the anti-nuclear factor test but negative in the L.E. cell test.

TABLE 2

Anti-nuclear factor in cases of Liver Disease

Diagnosis*	No. tested	Anti-nuclear factor positive
Post infective cirrhosis	9	4 (1 L.E.cell positive
Portal cirrhosis	4	1
Juvenile "	10	0
Alcoholic	4	0
Primary biliary cirrhosis	3	0
Other types of "	9	0

* Supplied by Dr. Dölle, Hammersmith Hospital.

TABLE 3

Titration of anti-nuclear factor

		titres of sera		
Diagnosis	No. tested	Neat - 1/10	1/20-1/100	1/100 - 1/300
S.L.E.	16	0	7	9
Rheumatoid Arthritis	13	1	10	2
Liver disease	4	0	4	0
Thyroid disease	6	1	5	0
Dermatomyositis	1	0	1	0
Pulmonary Tuberculosis	2	2	0	0
Normal sera (positive)	2	2	0	0
Totals:	44	6	27	11

Titration of anti-nuclear factor

Titration of anti-nuclear factor by serial dilution in saline was carried out in 44 cases, testing under standard conditions, using the same conjugate and tissue block. Table 3 gives the distribution of titres according to the diagnosis and shows that, except for two cases of rheumatoid arthritis, only sera from cases of systemic lupus could be diluted over 1/100 and still retain the ability to give a positive test. At the other end of the scale, the low titre of 1/10 was given by the 2 normal sera positives and the 2 pulmonary tuberculosis control group positives. In this group, too, came one case of thyroid disease and one case of rheumatoid arthritis. The majority of the rheumatoid arthritis and thyroid cases, and all the liver disease cases showed moderate activity.

Further analysis of the cases in the series

Certain clinical information and the results of routine investigations were obtained from the case notes of the patients with systemic lupus who attended the Rheumatism Unit at Taplow and at Hammersmith Hospital. These cases were seen by Professor Bywaters or the clinical staff attached to his unit. In addition, a group of 12 cases of rheumatoid arthritis with positive anti-nuclear factor tests, and a group of 40 cases with negative tests, again from the above units, were studied in the same way.

Table 4

Clinical and serological detail of the 20 cases of Systemic Lupus Erythematosis seen at Taplow and Hammersmith

	No. of cases	Percentage incidence
Sex distribution (females)	16	80
Duration of symptoms - greater than 5 years	10	50
Joint involvement (all types)	16	80
Hepatomegaly	5	25
Splenomegaly	6	30
Anti-nuclear factor positive	19	95
L.E. cell positive	15	75
Serum globulin - greater than 3G%	11	55
Serum albumin - less than 4G%	13	65
Elevated gamma globulin (electrophoresis)	14	70
Haemoglobin - less than 11G%	15	75
White cell count - less than 5000/cu.mm.	12	60
Rheumatoid factor (D.A.T.1/16 or more or Positive Latex test)	11	55
Wassermann test - positive	5	25
Platelet count (less than 100,000 per cu.mm.)	5	25

Table 5

Percentage incidence of certain manifestations of Systemic Lupus - Comparison of the present series with other published series

	present series (20)	Jessar et al. (323)	Dubois (62)	Shearn & Pirofsky (34)	Harvey et al. (105)	Copeland et al. (47)
Sex distribution females	80	85	89	91	78	93
Joint involvement types	80	77(44)	90	85	90	87
Hepatomegaly	25	29 "	34	44	32	43
Splenomegaly	30	27 "	8	41	15	19
L.E. Cells	75	-	69(60)	94(31)	82(96)	88(41)
Anaemia (Hb below 11G)	75	95 "	78	97	78	96
Leucopenia (1 count below 4,500/cu.mm.)	60 (less than 5,000)	70 "	68	74	68	57 (less than 4,000
Thromoctyopenia	25	-	10	31	26	8
Positive Wassermann Test	25	28 "	33	19	15	28

Note: figures in brackets = number of cases examined.

Table 6

Incidence of serological abnormalities, leucopenia and thrombocytopenia in the 19 anti-nuclear factor positive cases of Systemic Lupus Erythematosus

Coombs test Wassermann Cold agglutinins D.A.T. or latex Leucopenia Thrombocytopenia	Features shown by cases						
	1 or more	2 or more	3 or more	4 or more	5 or more	6	
	17	11	5	2	1	1	No. of cases
	89	58	26	10.5	5.2	5.2	Percentag

Systemic lupus erythematosus

Table 4 shows clinical and serological detail of a group of 20 cases of systemic lupus erythematosus. All but one of these cases was anti-nuclear factor positive and 15 (75%) were L.E. cell positive. Joint involvement (all types) was present in 16 (80%) of the cases, and 16 (80%) of the cases were females. Serum protein abnormalities were a common feature in the group, with elevation of the gamma globulin by electrophoresis in 14 (70%) of the cases. Three-quarters of the cases had a haemoglobin of less than 11 g.%. Leucopenia (white cell count of less than 5000/cu.mm.) was also a common feature. Five cases had a positive Wassermann test and 2 more were anti-complementary. Table 5 compares this series with other published series, which it resembles closely. Table 6 is a cumulative table of abnormal features in the 19 cases of systemic lupus which were anti-nuclear factor positive. The table shows that more than half the cases showed at least 2 of the features - Coombs test, Wassermann test, Cold agglutinins, rheumatoid factor (differential agglutination test (D.A.T.) or latex test), leucopenia or thrombocytopenia (less than 100,000 platelets/cu.mm.).

Rheumatoid arthritis

Table 7 shows the comparison of certain clinical, serological and haematological findings in the anti-nuclear factor positive group with those in the negative group. The results show that anaemia was significantly more common in the group with anti-nuclear factor activity,

and as might be expected, so also was the finding of L.E. cells. Other features with a X^2 approaching significance in the positive group were splenomegaly and low serum albumin. Table 8 is a cumulative table of cases showing various features and comparing the anti-nuclear factor positive systemic lupus group with the positive and negative rheumatoid arthritis groups. At least half the anti-nuclear factor positive cases, both in Systemic Lupus and Rheumatoid Arthritis, showed 4 or more of the features - hepatomegaly, splenomegaly, leucopenia, anaemia, low serum albumin, raised serum globulin positive L.E. cell test or positive test for Rheumatoid factor, compared with only 10% of the anti-nuclear factor negative rheumatoid arthritis group.

Discoid lupus

The patients included in this group were patients with typical lesions of discoid lupus erythematosus, in whom repeated examinations failed to reveal any systemic complications of their purely cutaneous syndrome. The cases, therefore, were classified by Drs. Scott and Rees who provided the sera into the category of group 4 cases which is defined as above (Scott and Rees 1959) in contrast to the other three groups which had systemic manifestations. A consideration of the severity of the disease (as estimated by Drs. Scott and Rees) in relation to the result of the anti-nuclear factor test, revealed a strikingly significant correlation.

TABLE 7

The serological and clinical findings in anti-nuclear factor positive and negative groups of rheumatoid arthritis patients.

Clinical features	No. of cases	% with feature	ANF +ve No. of cases	%	ANF -ve No. of cases	%	X^2
No. of cases	53		12		41		
Hepatomegaly	8	15	4	33	4	10	2.5
Splenomegaly	4	7.5	3	25	1	2.4	3.39
Serological features							
No. of cases	49		12		37		
Albumin less than 4 G%	26	53	9	75	17	46	3.158
Globulin greater than 3 G%	26	53	8	66	18	49	1.235
R.A.factor (D.A.T. or latex) No. of cases	110		12		98		
Positive RA factor	72	65	9	75	63	64	0.5
Blood findings							
Haemoglobulin No. of cases	62		12		50		
Less than 11 G%	21	34	8	66	13	26	6.71
White cells No. of cases	55		12		43		
Less than 5000/cu.mm.	5	9	3	25	2	4.6	2.05
L.E.cells No. of cases	86		12		74		
L.E.cell positive	4	4.6	3	25	1	1.3	7.4

TABLE 9

Comparison of severity of disease with presence and absence of anti-nuclear factor in Discoid Lupus

	Anti-nuclear factor positive	Anti-nuclear factor negative	Total
Mild	5	61	66
Severe	5	3	8
	10	64*	74

* one of the 65 anti-nuclear negative cases was not reported on clinically.

$X^2 = 18$

$X^2_y = 14$

TABLE 10

Comparison of features in anti-nuclear factor positive and negative cases of liver disease.

	L.E.cells	Globulin greater than 3g.%	Leucopenia less than 5000/cu.mm.	DAT 1/16	Anaemia less than11g.%	Joints	W.R.
Anti-nuclear Factor positive	1 20%	4 80%	3 60%	2 40%	3 60%	3 60%	2a/c -
Anti-nuclear factor negative	0 0%	18 81%	9 41%	0 0%	11 50%	2 9.5%	2a/c

Totals: Anti-nuclear factor positive : 5

Anti-nuclear factor negative : 22

TABLE 8

Cumulative Table of the Incidence of Selected Features in three Categories of Cases.

	No. of features								
	8	7	6	5	4	3	2	1	Total
Systemic Lupus Erythematosus Anti-nuclear factor positive	0	1	7	7	10	16	17	17	17
		6%	41%	41%	59%	94%	100%	100%	
Rheumatoid Arthritis Anti-nuclear factor positive	1	1	3	4	6	7	11	11	12
		8%	12%	33%	50%	58%	92%	92%	
Rheumatoid Arthritis Anti-nuclear factor negative	0	0	1	2	4	13	26	37	40
			2.5%	5%	10%	32.5%	65%	92.5%	

Features analysed:- Hepatomegaly, splenomegaly, leucopenia (less than 5000 per cu.mm.), anaemia (less than 11 g.%), serum albumin less than 4 g.%, serum globulin greater than 3 g.% , positive L.E. cell test and presence of rheumatoid factor (either DAT 1/16 or over, or positive latex test).

Liver disease

In Table 10 certain features of the 5 anti-nuclear factor positive liver disease cases were compared with those in 22 anti-nuclear factor negative cases of liver disease. Whilst the numbers were too small to draw definite conclusions, the higher incidence of joint manifestations and the presence of rheumatoid factor in the anti-nuclear factor positive liver cases was an interesting feature of the group.

CHAPTER 3

E X P E R I M E N T A L

A. STUDIES ON THE NATURE OF THE "ANTIBODY"

1. Physicochemical

The major components of human serum as determined by electrophoresis and immunoelectrophoresis are shown on plate 6. This shows that gamma globulin can be separated electrophoretically from all the other serum components. Immunoelectrophoretic studies of the reaction between human serum and anti-human serum have shown that only one precipitin band appears in the gamma globulin area. The isolated reports of a second band in this region are considered to be due to denatured gamma globulin (Crowle 1961). The beta globulin component of serum consists of two main components, $beta_1$ and $beta_2$. An important $beta_2$ globulin is that known as $beta_{2M}$ or macroglobulin, which has a sedimentation constant of 19 S and contains the human iso-agglutinins. The gamma globulin component has a sedimentation constant of 7 S. The $beta_2$ and the gamma globulin are collectively called the "immunoglobulins" because almost all of these appear to be some form of antibody (Crowle 1961).

The following studies report the result of fractionation procedures designed to isolate the gamma globulin component of sera and to determine if anti-nuclear factor be present in this fraction or in the $beta_{2M}$ macroglobulin fraction, or in both fractions.

Plate 6.

DIAGRAM SHOWING MAJOR SERUM COMPONENTS

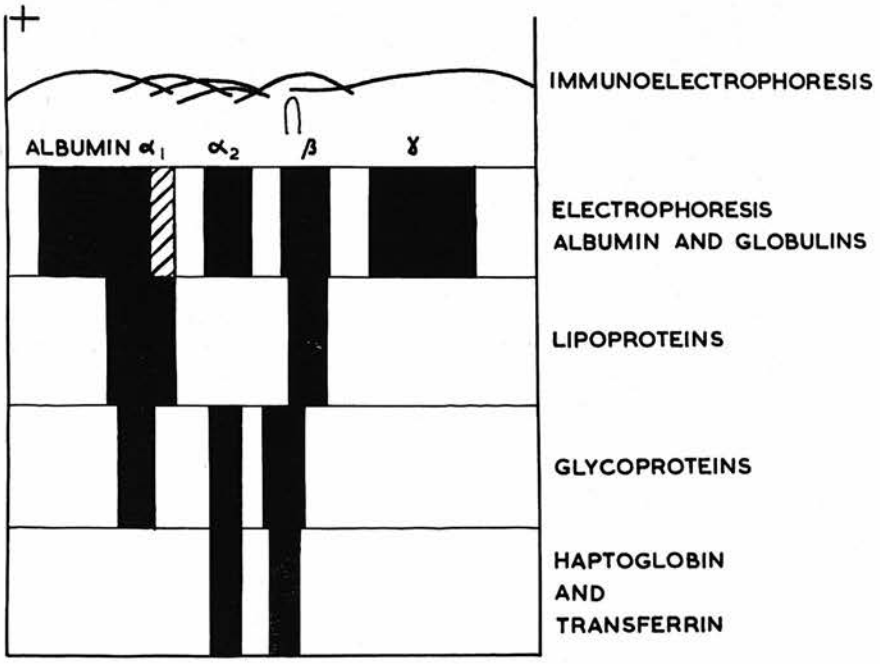
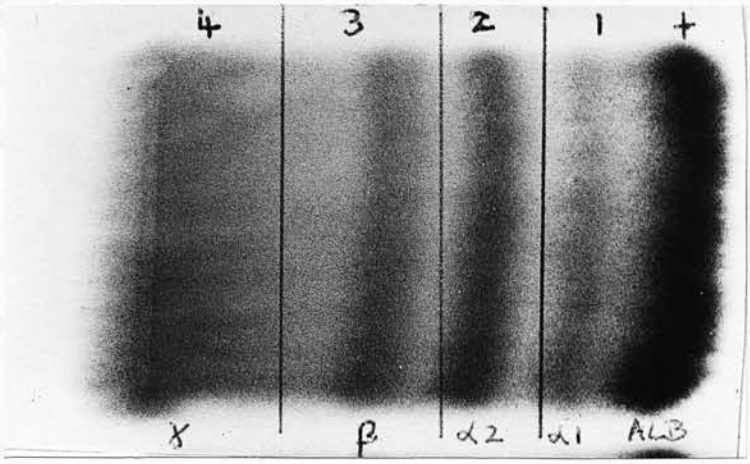


Plate 7.

Electrophoresis of human sera on thick electrophoresis paper.



The vertical lines indicate where the paper was cut.

Salt Precipitation

Crude fractionation by salt precipitation was carried out on two high titre anti-nuclear factor positive sera from patients with systemic lupus erythematosus (SLE serum H, and SLE serum P) and one low titre serum from a case of liver disease. The procedure was as follows:-

Specimens of the sera were chilled in the ice compartment of the refrigerator until ice crystals were beginning to form. An equal volume of pre-cooled saturated ammonium sulphate was added, the mixture was shaken thoroughly and then left in the cold (4°C.) for 2 hours. The precipitate was recovered by rapid centrifugation in the cold in an M.S.E. major refrigerated centrifuge at 3000 r.p.m. for 45 minutes, dissolved in distilled water and stored in the cold. Electrophoresis of this solution at 150 volts for 2 hours (Veronal buffer pH.8.6) showed that the preparation contained gamma globulin together with smaller amounts of the other serum fractions. The solution was shown to contain anti-nuclear factor activity by the fluorescent antibody test using infant thyroid sections as previously described.

Fractionation of serum on thick electrophoresis paper.

Thick electrophoresis paper (Whatman No. 17) was used in order to absorb sufficient serum. Approximately 0.02 ml. of each of the three sera used in the previous experiment was applied to the paper and electrophoresis was carried out for $2\frac{1}{2}$ hours at 150 volts in veronal buffer pH 8.6. Each serum was run in duplicate and one electrophoretic

strip was dried and stained with Ponceau S (Gurr) in order to localise the various fractions of the serum. Using thestained strip as a guide, the undried parallel strip was cut into four parts as shown in plate 7. This shows a paper electrophoresis strip with lines drawn where the paper was cut in the experiment. Part one contained the albumin and alpha$_1$ globulin, part two the alpha$_2$ globulin, part three the beta globulin and part of the gamma globulin, and the final portion contained the main gamma globulin fraction. Each of these pieces of paper was placed over an air dried film made from the buffy coat of human blood and the protein was eluted on to the film with 0.1 ml. of phosphate buffered saline pH 7.0. After 30 minutes at 37°C. the films were washed and stained with fluorescein conjugated anti-human globulin serum. Examination under the fluorescence microscope for fluorescent staining of the nuclei showed the following results:-

TABLE 11

	Albumin and alpha$_1$	Alpha 2	Beta and gamma	Gamma	Whole serum
S.L.E. serum H	-	-	+	++	+++
S.L.E. serum P	-	-	-	++	++
Liver disease serum Hu	-	-	-	-	+

+++) indicates
++) bright staining
\+ indicates weak staining
\- indicates absence of staining

These results show that the anti-nuclear activity is present in the gamma globulin component of the two S.L.E. sera. The weak nuclear staining in the beta plus gamma globulin fraction of serum could be due entirely to the gamma globulin content or it could be due to some component of the beta globulins. The preparation made from the liver disease serum failed to stain the nuclei after treatment. The explanation for this was found in a later study (thiol study) when it was found that this serum contained its anti-nuclear activity in the macroglobulin fraction (which would not be eluted from the paper - Consden personal communication).

Fractionation of serum on anion exchange resin

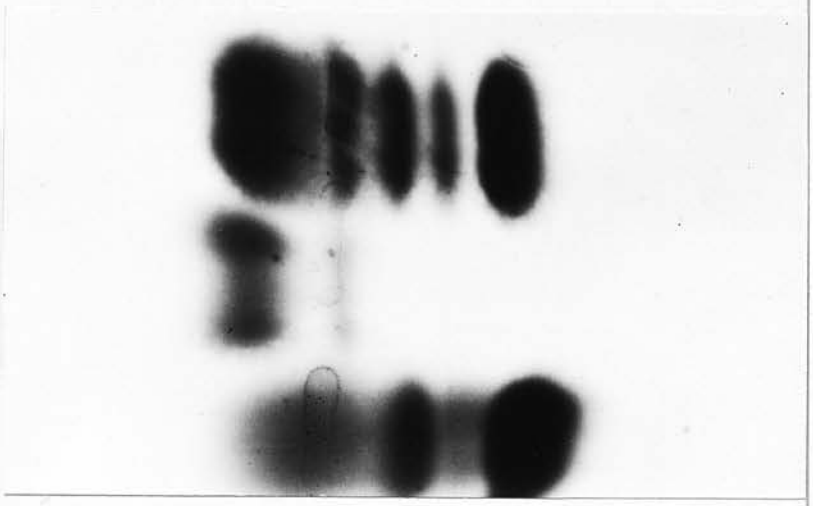
The method of Levy and Sobers (1960) was used to obtain pure gamma globulin from two sera containing anti-nuclear factor (S.L.E. serum H and Rheumatoid Arthritis serum Ha), and from one normal control serum. Whatman D.E . 50 powder (the exchanger) was washed three times with 0.5 N NaOH, finally re-suspended in water and washed to neutrality. The slurry (sediment) was adjusted to pH 6.3 with 0.2 M. NaH_2PO_4 and washed several times with the starting buffer - 0.0175 M Sodium Phosphate (pH 6.3). This preparation was stored in the cold until required and was then gently re-suspended and the slowly sedimenting "fines" discarded. For 2 ml. quantities of serum an exchanger column of 5 cm. by 1 cm. was used in a tube with a small wad of glass wool and a tap at the bottom to prevent the exchanger running out. Before use, the column was washed

with at least 50 mls. of the starting buffer and the pH of the buffer which had passed through checked to be 6.3. At no time was the column allowed to dry, the fluid level being kept above the surface of the exchanger. The serum for fractionation was dialysed overnight against the starting buffer using 100 to 200 volumes of buffer for each volume of serum. After dialysis the serum was added to the top of the column which had been run down so that the level of the buffer was just above the exchanger. The serum was then washed through the column with 20 ml. buffer so that the gamma globulin would pass through the column, and the other components (including the macroglobulins) would be held back in the column. These other components were subsequently washed through with 2 M NaCl in 0.4 M Na phosphate. The resulting fractions were concentrated to near the original volume by introducing carbo wax into a piece of dialysis tubing and placing this tubing in a beaker containing the fraction, leaving the open end of the tubing hanging outside the beaker. The final solutions of concentrated fractions dialysed against N saline in order to adjust the ionic strength for use in serological tests. The fractions were stored at -20°C. Electrophoresis of the fractions was carried out to ascertain the result of the fractionation process (plate 8). In the case of serum H and serum Ha, the "gamma globulin" fractions were shown to contain anti-nuclear activity by the fluorescent antibody technique. The normal control was negative. Electrophoresis of these "gamma globulin" fractions showed that they consisted largely of gamma globulin, but did contain a trace of beta globulin at the origin.

Plate 8.

Electrophoresis on cellulose acetate (Veronal buffer ph 8.6, 150 volts. 2 hours) of the fractions obtained by cellulose column chromatography of an anti-nuclear factor positive serum (S.L.E. serum H).

+



Whole serum

"gamma globulin"

"remaining fraction"

Note trace of beta globulin in "gamma globulin" fraction and most of the other serum components in "remaining fraction".

Plate 9.

Immunoelectrophoresis of "gamma globulin" fraction obtained by D.E.A.E. chromatography.

Note well marked gamma globulin band and smaller band opposite the beta globulins.

Immunoelectrophoresis of this fraction against anti-human serum (carried out as described in Chapter 1) confirmed this finding. Plate 9 shows one main gamma globulin band and a smaller band opposite the beta globulins.

These findings by column fractionation are the same as those on S.L.E. H serum when fractionated on thick electrophoresis paper. Goodman et al. (1959,1960) have shown that macroglobulins occur in the "remaining fraction". The "remaining fractions" of sera H and Ha each show anti-nuclear activity by the fluorescent antibody technique, but the localisation of this activity is not possible since this fraction, in addition to the macroglobulins already mentioned, also contains all the other serum components, including some gamma globulin (plate 8).

Ultracentrifugal studies

The high molecular weight beta 2 M globulins (19S Macroglobulins) sediment in the ultracentrifuge more rapidly than the other serum components. In the model E Spinco using the number 40 rotor at 40,000 r.p.m. (144,700G), it is possible to clear the macroglobulins completely from the supernatant layers after 4 hours centrifugation (Schachman 1959). Thus at 4 hours the macroglobulins will all be in the lowest layer, whilst the other more slowly sedimenting components, and in particular the 7 S gamma globulin fraction, will still be spread out in the upper layers.

Table 12

Ultracentrifuge Studies

	Fraction No.	Anti-nuclear factor	L.E. cell factor	Anti-nuclear staining after Mercaptoethanol
S.L.E. serum H.	1	+++	+	
	2	+++	+	
	3	+++	+	Unaffected
	4	+++	+	- 18 L.E.cells/500wbc
	5	+++	+	- 17 " /500 wbc
S.L.E. serum F.	1	-	-	
	2	+	-	
	3	++	-	Unaffected
	4	++	-	
	5	+++	-	
R.A. serum T.	1	-	-	
	2	tr.	-	
	3	+	-	Unaffected
	4	+	-	
	5	++	-	
R.A. serum L.	1	+	-	
	2	++	-	
	3	++	-	
	4	++	-	Unaffected
	5	+++	-	
R.A. serum S.	1	-	-	
	2	+	-	
	3	+	-	reduced
	4	++	-	
	5	+++	-	
R.A. serum M.	1	-	-	
	2	tr.	-	
	3	+	-	reduced
	4	+	-	
	5	++	-	
R.A. serum Ha.	1	-	-	
	2	-	-	
	3	tr.	-	reduced
	4	+	-	
	5	++	-	

Anti-nuclear staining
+++ indicates strong reaction
++ indicates moderate "
+ indicates weak "
tr. indicates trace.

L.E. cells
+ indicates positive test
- indicates negative test

This procedure was performed on 2 sera from patients with Systemic Lupus (sera H and F) and 5 sera from patients with Rheumatoid Arthritis (T, L, S, M and Ha.). Twelve ml. samples of sera diluted 1/2 with saline, were pipetted into plastic cups and centrifuged at the above speed for 4 hours. On examination of the centrifuged specimens, 5 layers could be distinguished; these were removed successively with a Pasteur pipette and stored in separate containers at -20°C. The anti-nuclear factor activity of the various fractions was tested by the fluorescent antibody method and the results are shown on table 12; (the final column contains the results of the next experiment). With each of the sera anti-nuclear activity was found to be present in the supernatant layers, as well as in the lowest layer (No.5). The L.E. cell factor, being 7 S globulin, is spread throughout the layers. It can therefore be concluded that in the sera tested, the anti-nuclear factor is present in serum fractions other than the macroglobulin, although it may be present in the macroglobulin fraction in addition. Chemical tests were carried out to provide further information on this point.

The use of a thiol reducing agent to distinguish 19 S macroglobulin from 7S gamma globulin

In the previous experiment, layer 5, containing the macroglobulins together with other serum components, showed anti-nuclear activity. In order to show if any of this activity was due to the macroglobulins, the sera were treated with the thiol reducing agent Mercaptoethanol. This sulphydryl compound has been shown to split macroglobulin antibodies into

serologically inactive components by breaking the S-S bonds and forming 2 SH groups (Deutch and Morton 1957, Grubb and Swahn 1958).

All the sera which were used in the ultracentrifuge experiment were included in this study and in addition 17 other sera including S.L.E. serum P and Liver HU which were used in the electrophoresis elution test. Mercaptoethanol (Light's) was made up into an 0.2 M solution in phosphate buffer pH 7.4 (0.2M KH_2PO_4 K_2HPO_4). Sera were diluted 1/2 with buffer, an equal part of 0.2M mercaptoethanol was added and the tubes stored in the fume cupboard for 48 hours. Control tubes were set up containing serum and buffer only. The solutions were then dialysed for 72 hours at 4°C. against phosphate buffer containing iodoacetamide (0.02M) to prevent re-combination of the globulin components. The treated and control specimens were tested for anti-nuclear factor activity by the fluorescent antibody method, in the usual way except that iodacetamide buffer was used for washing the sections instead of saline. Table 12 shows the results of the mercaptoethanol treatment on the anti-nuclear activity of the sera used in the ultra-centrifuge experiment. The two Systemic Lupus sera were unaffected by the treatment, as were two of the Rheumatoid Arthritis sera , whereas the staining in the remaining 3 Rheumatoid Arthritis sera was reduced. Thus the unaffected sera contain their anti-nuclear activity predominantly in the non-macro immunoglobulin (7 S gamma globulin). The sera which had their activity reduced by mercaptoethanol and gave staining with the macroglobulin-free

ultracentrifuge fractions, contained their activity in both fractions.

Table 13 shows the results of the anti-nuclear factor test on all the 25 sera after mercaptoethanol treatment. Six more Systemic Lupus sera were unaffected indicating that anti-nuclear factor in this disease is predominantly non-macroglobulin in type. A total of three Rheumatoid Arthritis sera were unaffected and three were rendered free of anti-nuclear staining. In Rheumatoid Arthritis, therefore, the activity can be 7 S or 19 S or a mixture of the two. Serum Hu was the one liver disease case affected by mercaptoethanol and this confirms the previous result on electrophoresis paper which indicated that the activity remained on the paper as would be expected if the activity was due to macroglobulin. In addition, confirmation that the anti-nuclear activity of the "remaining fraction" of S.L.E. serum H taken off the D.E.A.E. cellulose column (which contains both 19 S and 7 S globulin) was not due to its macroglobulin content, was obtained by the failure of mercaptoethanol to affect its activity. Finally the L.E. cell activity of S.L.E. serum H was shown to be unaffected by mercaptoethanol, confirming that it was 7 S globulin.

Table 13

Result of Mercaptoethanol treatment of 24 anti-nuclear factor positive sera.

	Total tested	Anti-nuclear factor positive	Anti-nuclear factor negative
Systemic Lupus Erythematosus	8	8 unaffected	0
Rheumatoid Arthritis	9	* 6	3
Liver Disease	3	2 unaffected	1
Thyroid Disease	2	1 unaffected	1
Dermatomyositis	1	1 unaffected	0
Pulmonary tuberculosis and drug sensitivity	1	0	1
	24	18	6

* 3 unaffected and 3 reduced.

Relationship of globulin type to the nuclear antigen

Absorption studies with nuclear materials were carried out on a group of 9 anti-nuclear factor positive sera in the non-macroglobulin category and with 3 sera in which anti-nuclear activity had been shown to be macroglobulin in character. The results are shown in table .

Table 14

Comparison of globulin type with result of absorption with nucleoprotein

Anti-nuclear factor	No. of cases showing absorption with calf thymus nucleoprotein		Total tested	Diagnosis
	complete	incomplete		
Non Macroglobulin	2	2	4	Systemic Lupus
	1	1	2	Liver Disease
	2	0	2	Rheumatoid Arthritis
	1	0	1	Thyroid Disease
Macroglobulin	1	0	1	Rheumatoid Arthritis
	1	0	1	Liver Disease
	0	1	1	Thyroid Disease

These results show that there is no relationship between the globulin nature of the anti-nuclear factor and the effect of nucleoprotein absorption.

2. Study of precipitation reactions between anti-nuclear factor and nuclear materials

The experiments to be described include qualitative and quantitative precipitin tests with calf thymus nucleoprotein and qualitative immunodiffusion and tube precipitin tests with D.N.A. and histone.

Precipitation tests with calf thymus nucleoprotein

For this work nucleoprotein prepared from fresh calf thymus as described in the chapter on material and methods was used to detect possible precipitation reactions with anti-nuclear factor positive sera. The nucleoprotein was dissolved in 1 M. NaCl and contained 1.2 mg/ml. of D.N.A. and 1.2 mg/ml. of protein. Forty-five sera were used in this study as shown in table 15A. Two sera showing marked rise in gamma globulin on electrophoresis were included in view of the increase in gamma globulin found in many S.L.E. sera. The sera were cleared by centrifugation in the high speed attachment of the M.S.E. major centrifuge at about 10,000 r.p.m. for 30 minutes.

Capillary tube precipitation tests

For this test the viscous nucleoprotein solution was diluted 1/2 with 1 M. NaCl as it was found that mixing of the serum and undiluted nucleoprotein failed to take place in the capillary tubes. The tests were set up in the classical manner, the serum being drawn up into the capillary tubes followed by the nucleoprotein. The tubes were

inserted into plasticine on wooden racks and incubated at 37°C. for two hours. Controls were set up for each serum with 1 M. saline instead of the nucleoprotein solution, and with nucleoprotein and saline instead of serum. Observation of the tubes during the incubation period showed precipitate formation in some of the tubes, beginning as an opaque layer at the interface due to coating of the more viscous nucleoprotein. As the nucleoprotein penetrated into the column of serum, the appearance gradually changed to coating of the "coil" of nucleoprotein followed by fragmentation and precipitate formation.

At the end of the 2 hour incubation period, precipitate formation was recorded as follows:-

TABLE 15A

A.

Estimate of Precipitate

	Precipitate formation at two hours			
	Moderate-Strong	weak	negative	
S.L.E.	6	2	12	Anti-nuclear factor positive
Rheum. Arthr.	3	-	8	
Liver disease	-	2	2	
Normal sera	-	-	8	Anti-nuclear factor negative
Hypergammaglobulin sera	-	-	2	

The controls with serum and 1 M. saline, and those with nucleoprotein and saline were all negative. The tubes were then placed in the refrigerator and observed at intervals. It was found that gradually over the next 48 hours precipitate formation occurred in most of the

serum nucleoprotein tubes as shown on table 15B :-

TABLE 15

B.

Estimate of Precipitate

Precipitate formation at forty-eight hours

	Moderate-Strong	weak	negative	
S.L.E.	6	14	-	Anti-nuclear factor positive
Rheum. Arthr.	5	5	1	
Liver disease	-	3	1	
Normal sera	-	3	5	Anti-nuclear factor negative
Hypergammaglobulin sera	-	2	-	

The more rapidly formed precipitates (listed in table 15 A) were, in general, much heavier than those which appeared after the 2 hour incubation period, and this was confirmed by the quantitative study described in the next section.

Tests of the effect on precipitate formation of dilutions of the nucleoprotein solution were carried out with four of the nucleoprotein precipitating sera. The results after the 2 hour incubation period were as follows:-

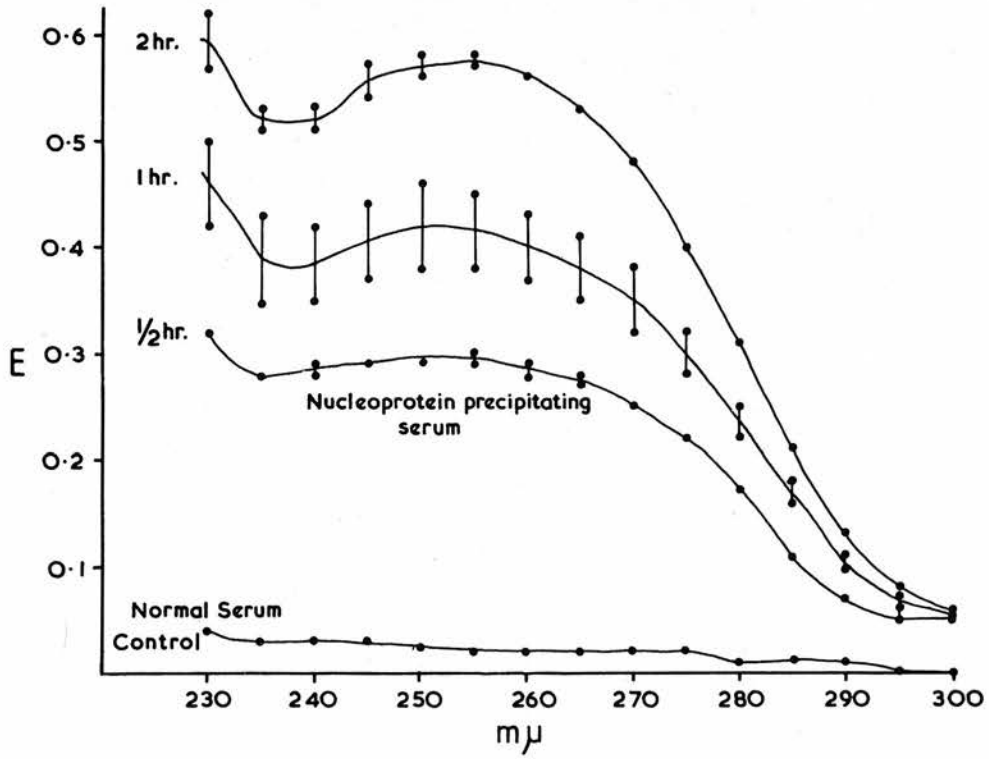
TABLE 16

	Concentration of nucleoprotein solution mg/ml.			
	1.2	0.6	0.3	0.15
Serum 1	+++	++	+	-
2	++	+	-	-
3	+++	+	-	-
4	+	-	-	-

+++ indicates strong precipitation
++ indicates moderate precipitation
+ indicates weak precipitation

These results show no evidence that antigen excess was achieved (i.e. at higher antigen concentrations the amount of precipitate should be less due to the formation of soluble antigen-antibody complexes). The possibility that antigen excess is not achieved under these conditions due to the high viscosity of the nucleoprotein solution is the most likely explanation of these results (see next section).

Figure 2. Absorption curves from 230 mμ to 300 mμ obtained with nucleoprotein-serum precipitates at ½ hour, 1 hour and 2 hours.



In figure 2- 7,
Vertical lines join the readings obtained from the 2 samples of S.L.E. H Serum.

Figure 3. Absorption curves from 230 mμ to 300 mμ obtained with nucleoprotein-serum precipitates at 4 hours, 6½ hours. 24 hours and 48 hours.

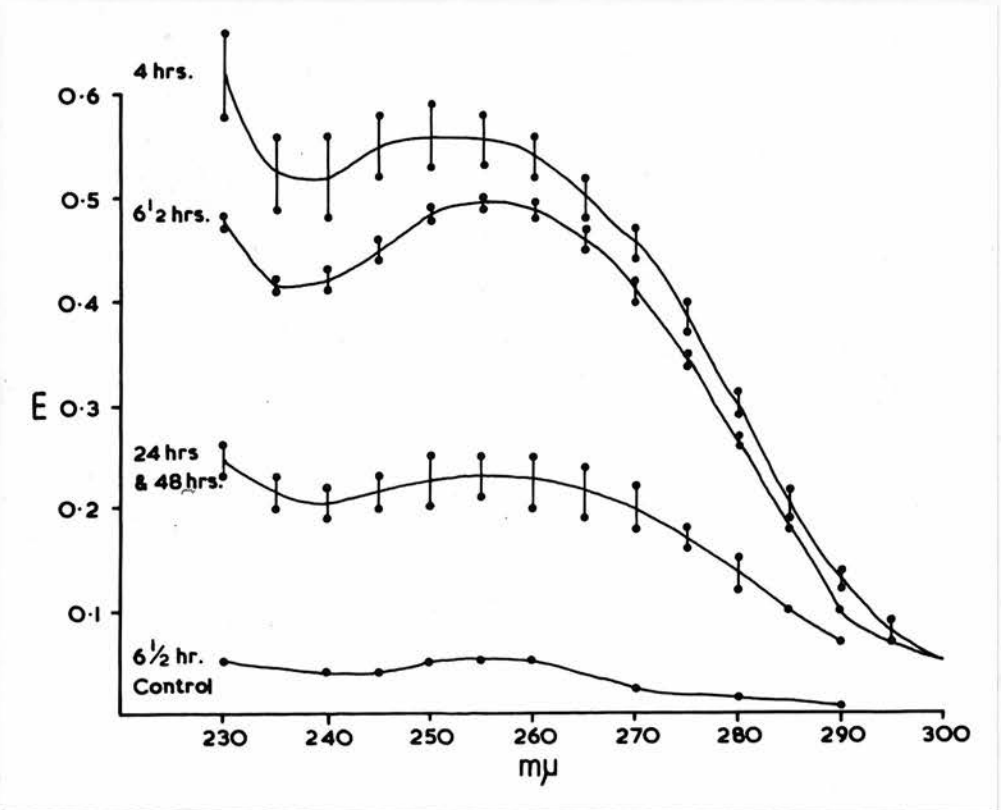
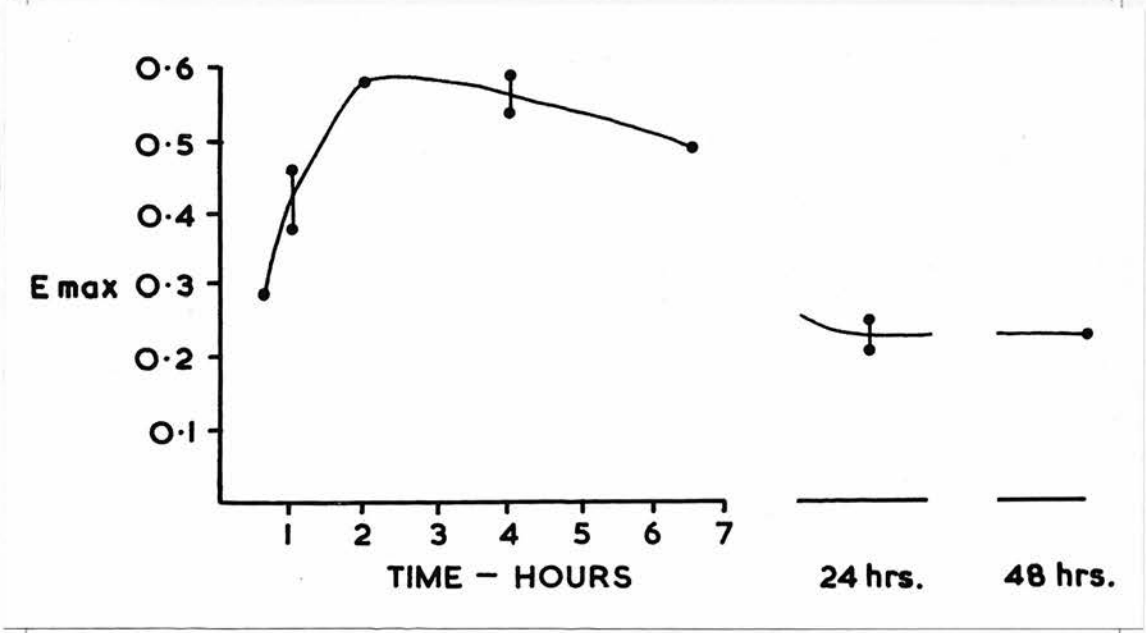


Figure 4.

Curve of maximal absorptions with nucleoprotein-serum precipitates at the hours indicated, derived from Figs. 2 and 3.



Quantitative precipitation tests with Nucleoprotein and Systemic Lupus sera

Spectrophotometric estimation of D.N.A. in the precipitate was used in the present study which was undertaken in collaboration with Dr. R. Consden.

In order to determine when maximum amount of precipitation occurred, precipitates were prepared from the interaction of nucleoprotein and Systemic Lupus sera at intervals up to 48 hours as follows:-
0.2 ml. of undiluted serum placed in each of 6 pairs of tubes, and 0.2 ml. of buffer (0.5M. $NaHCO_3.Na_2CO_3$, pH 8.0) and 0.2 ml. of calf thymus nucleoprotein solution (0.6 mg. per ml.) were then added. A drop of toluene was introduced as a preservative. The tubes were thoroughly shaken and incubated at 37°C. A normal serum control was set up in a duplicate series. At intervals of ½, 1, 2, 4, 24, and 48 hours, a pair of tubes was removed from each of the test and normal groups, and the precipitate brought down by centrifugation at 2,000 r.p.m. in an M.S.E. minor centrifuge for 5 minutes. The precipitate was then washed twice in normal saline and then in distilled water, and then dissolved in 5 ml. of N/10 NaOH. The D.N.A. absorption was then measured in a Unicam spectrophotometer over the range 230mμ to 300mμ. The maximum absorption was obtained at about 250mμ which is slightly lower than the absorption for D.N.A. itself (260mμ). It was considered that this was due to the tyrosine of the protein in the precipitate. The amount of precipitate from the normal serum control was found to be approximately 10 times less than that from the test serum. (Figs. 2 and 3).

Comparison of the results at these intervals showed that the maximum quantity of precipitate was formed between 2 and 4 hours (Fig. 4). The amount of precipitate was measured at $2\frac{1}{2}$ hours with varying quantities of nucleoprotein in order to construct a precipitin curve for this system, and to compare its characteristics with those obtained in other well-established antibody antigen systems. The method used was as above, using the following quantities:-

0.1 ml. of test serum and 0.1 ml. of buffer were added to three tubes containing 0.1 ml. of undiluted, 1/4, and 1/16 respectively of nucleoprotein solution.

The curve for the maximal absorptions at each dilution of nucleoprotein is shown on Fig 5 . This curve does not follow the classical pattern of decrease in the quantity of precipitate in the presence of excess antigen.

The possibility arose that this was due to the fact that antigen excess was in fact never achieved because the undue viscosity of nucleoprotein made it difficult to ensure adequate mixing. To avoid this difficulty the system was reversed and a constant relatively unviscous nucleoprotein concentration of 1/4 of the stock solution was added to varying dilutions of the serum. The precipitates were prepared as above and the absorptions measured similarly. The results are shown in Fig. 6 When these results are represented in terms of the concentration of nucleoprotein per unit of antibody (i.e. 50% dilution of serum contains twice as much nucleoprotein per unit of antibody as undiluted serum) and

Figure 5.

Curve showing maximal absorptions obtained with nucleoprotein-serum precipitates with increasing nucleoprotein concentration and constant serum (at $2\frac{1}{2}$ hours)

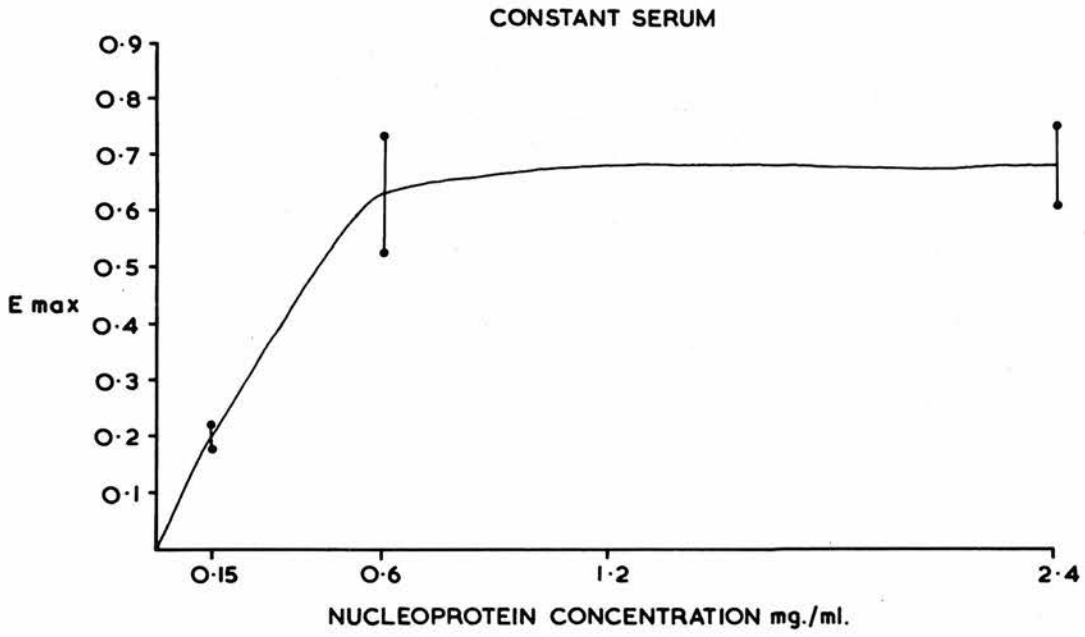


Figure 6.

Curve of maximal absorption obtained with nucleoprotein-serum precipitates with increasing serum concentration and constant nucleoprotein. (at $2\frac{1}{2}$ hours)

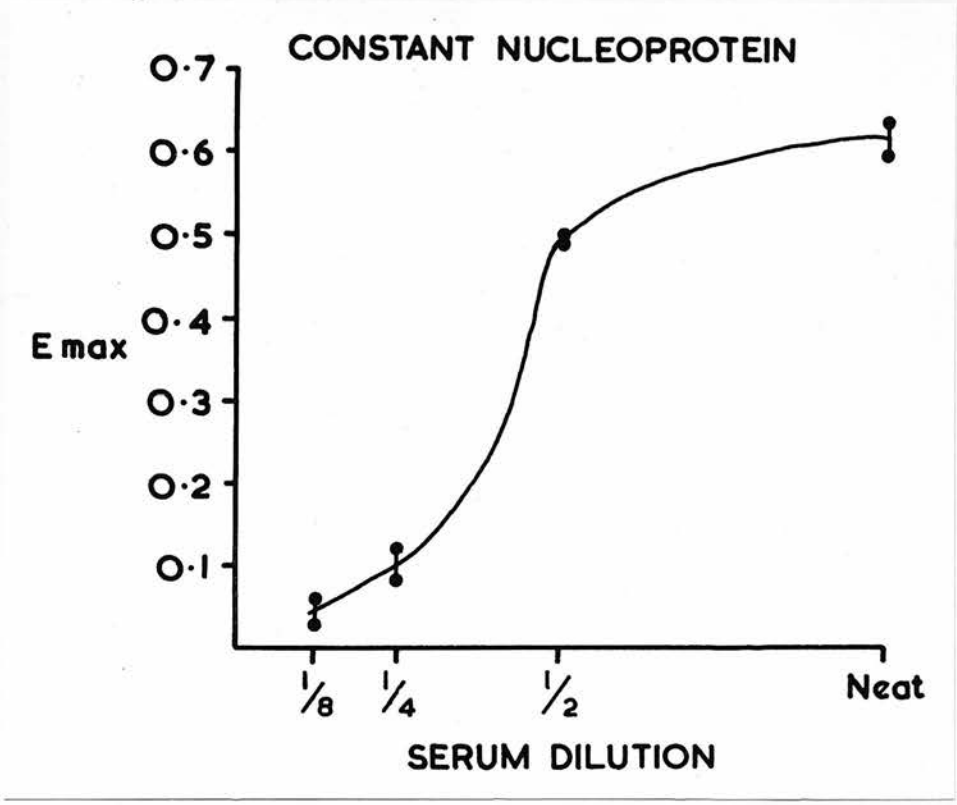
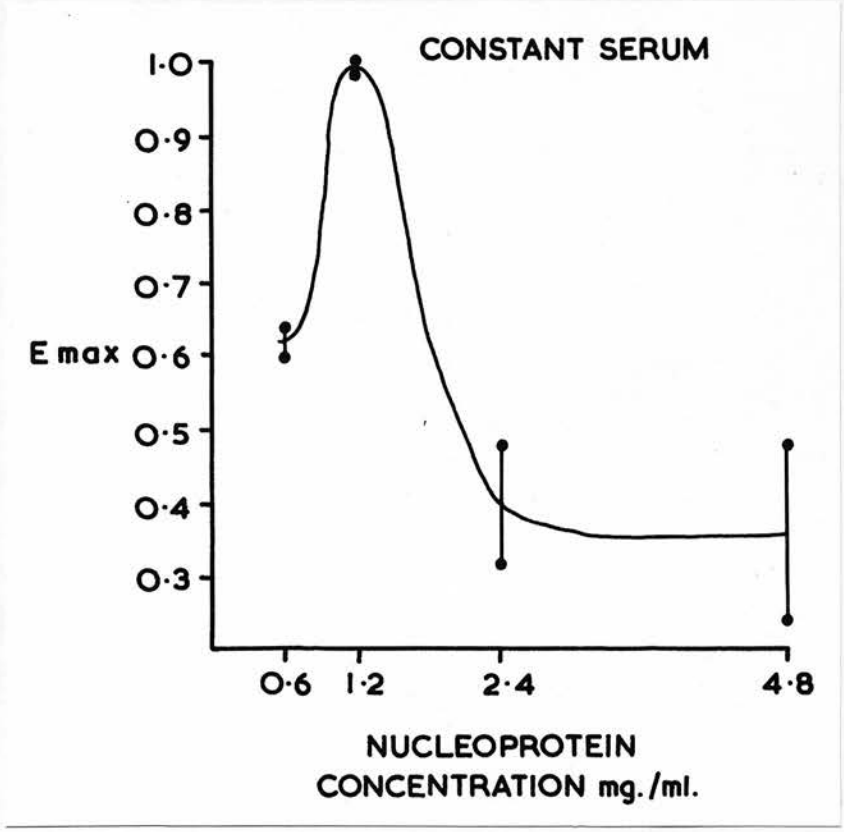


Figure 7

Curve of maximal absorptions of nucleoprotein-serum precipitates with constant serum and increasing nucleoprotein concentration (derived from fig. 6).



Note decrease in quantity of precipitate with the higher concentrations of nucleoprotein.

the amount of precipitate is calculated in terms of undiluted serum, a curve of the classical type can be drawn, showing decrease in the quantity of precipitate with increase in the antigen concentration (Fig 7).

Note: Other ways of overcoming the difficulty of the hyperviscosity of the nucleoprotein were attempted using the following depolymerisation methods:-

(a) Exposure to an equal volume of N/10 NaOH for 15 minutes at 20°C.

(b) Exposure to DNA'ase 0.5 mgs. per ml. in 0.003M. $MgSO_4$ for 30 minutes at 37°C.

(c) Sonic disintegration at 20 kilocyles/sec. for 18 minutes in an ice bath.

These procedures so reduced the precipitating capacity of the nucleoprotein that only weak positive or negative reactions were obtained.

Immunodiffusion on cellulose acetate paper

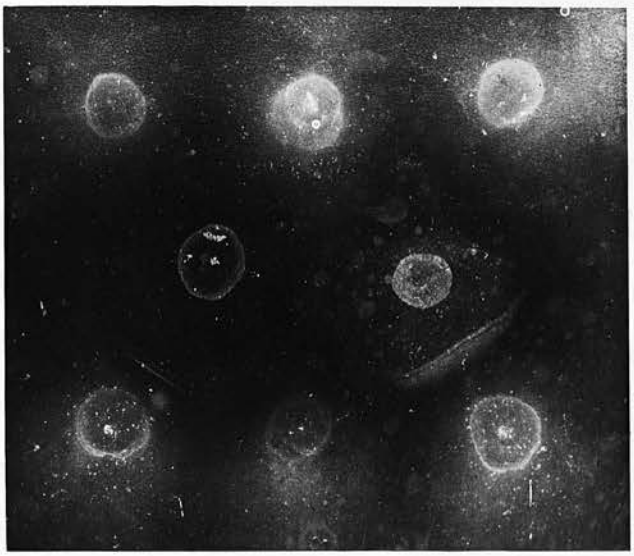
The cellulose acetate immunodiffusion method of Consden and Kohn (1959) was used as follows:-

Oxoid cellulose acetate paper was cut into 4 cm. x 4 cm. squares and washed for 10 minutes in phosphate buffered saline (pH.7.2). The paper was then blotted and the intended positions of the reactants marked with a pencil, placing the antigen and the antibody 1 cm. apart. The square was placed on a supporting stand consisting of pins embedded point upwards in plasticine. The antigen and antibody were placed on

the marked areas, using a capillary tube, and allowed to soak into the paper. The paper was then immersed in liquid paraffin and left for 48 hours at room temperature for diffusion to take place. It was then washed in petrol to remove the liquid paraffin and in the buffered saline (4 hours) to remove the unreacted proteins. The paper was stained with Ponceau S (Gurr) (0.15% in 3% trichlor acetic acid) for 5 minutes, washed in 5% acetic acid and then stained with Nigrosine (0.0025% in 2% acetic acid) for 18 hours. The excess stain was washed out in running tap water. In this experiment calf thymus nucleoprotein was used at a concentration of 2.4 mg. per ml. in 1 M. saline. Two preparations were used, one of which had been sonerated as previously described and the other untreated. Of two S.L.E. sera which had given strong precipitates in capillary tubes with nucleoprotein, one gave a precipitation line when tested on cellulose acetate. The line was more marked with the unsonerated specimen as would be expected (plate 10). The other S.L.E. serum and the two normal control sera showed no precipitation lines.

Plate 10.

Cellulose acetate immunodiffusion test showing precipitin line between calf thymus nucleoprotein and serum from systemic lupus patient.



L.E. serum (2) Normal serum L.E. serum (2)

Nucleoprotein (sonerated) Nucleoprotein (unsonerated)

L.E. serum (1) Normal serum L.E. serum (1)

Precipitation tests with D.N.A.

(a) Capillary tube precipitation tests.

Nine different anti-nuclear factor positive sera were used in these tests with normal control sera in each experiment. Calf thymus nucleoprotein (Worthington Biochemical Corporation) was used as the antigen and as this is insoluble in physiological saline it was treated with N/10 NaOH for a few seconds to enable the preparation to go into solution in phosphate buffered saline pH 7.0. The D.N.A. solution was made up at a concentration of 1 mg/ml. Capillary tube precipitin tests were set up with the D.N.A. solution made up in doubling dilutions from neat (1 mg/ml.) to a dilution of 1/1024 (approx. 1µg./ml.). The tests were uncubated at 37°C.for 2 hours and then at 4°C. for five days. Examination of the tests at 24 hours and 48 hours showed no evidence of precipitate formation. At five days, slight non-specific precipitate formation was present in all the tests including the controls.

(b) Ouchterlony double diffusion tests in agar plates.

Agar was made up at concentrations of 0.5 and 1% in phosphate buffered saline pH 7.0 and 10 ml. of the heated solution was poured into clean Petrie dishes. After the agar had solidified, a pattern of wells was cut with a Feinberg qualitative cutting template (Shandon) and the agar plugs removed by suction. The pattern consisted of a central well with six slightly smaller peripheral wells, each situated 1 cm. from the central well. In view of the large size of the D.N.A.

molecule (1,000,000 to 3,000,000 M.W.) and the possible effect of this on its ability to diffuse in agar, quantities of the D.N.A.were exposed to heat with the intention of depolymerising the molecules as follows:- Heating in a water bath at 80°C. and 100°C. for ½, 1 and 2 minutes (longer exposure to heat e.g. 20 minutes at 100°C. has been shown to reduce the viscosity of a D.N.A. solution to 4% of the initial value and results in a preparation which will not precipitate with S.L.E. sera - (Barbu,Seligmann and Jolly)(1960).

The D.N.A. solutions were placed in the central wells of a series of plates and the test sera were placed in the peripheral wells. Incubation of the plates for 14 days at 37°C., examining the plates daily, showed no evidence of precipitate formation in any of the preparations.

(c) Immunodiffusion on cellulose acetate paper.

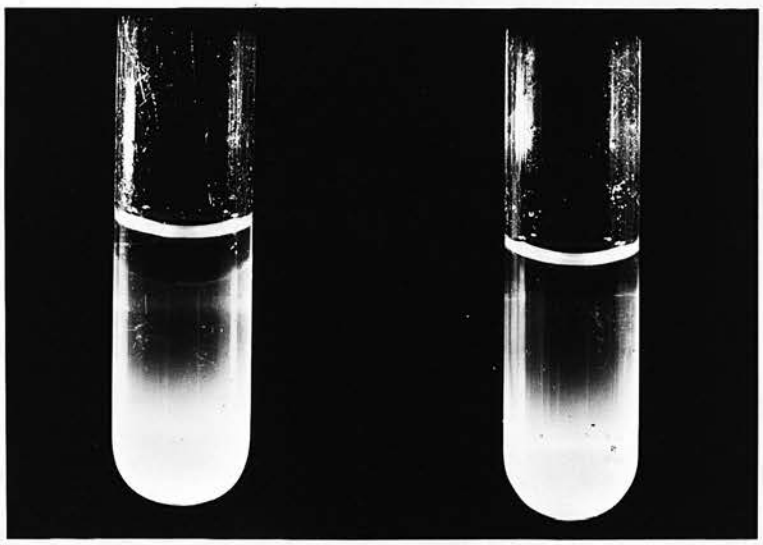
The anti-nuclear factor positive sera were tested against the D.N.A. preparation on cellulose acetate paper using the method described for the nucleoprotein experiments. No precipitin lines were observed on stained preparations which had been allowed to diffuse under liquid paraffin for 24, 48 and 96 hours.

(d) The Preer (1956) modification of the Oakley Fulthorpe (1953) double diffusion technique in tubes.

This technique is one of the most sensitive methods for detecting precipitins and can detect 0.03 µg to 0.09 µg. of antibody

Plate 11.

Double diffusion test in agar showing precipitin band between D.N.A. and serum of a patient with systemic lupus.



Positive test Normal serum

nitrogen (Kabat and Mayer 1961). The test was carried out using 3" x $\frac{3}{8}$" glass tubes which had been coated on the inner surface with a layer of 0.05% agar in 0.85% saline containing 1/10,000 merthiolate, dried and stored in a dessicator. Using a capillary pipette, 0.15 ml. of undiluted serum was introduced into the bottom of the tubes, care being taken to avoid touching the side of the tube. The tubes were placed in an ice bath (to allow rapid solidification of the agar) and 0.3 ml. of the 0.5% agar solution was carefully layered on top of the serum. Finally, 0.15 ml. of D.N.A. solution (0.5 mg/ml. in deionised water) was layered above the agar and the stoppered tubes kept at 37°C.

Sera from 20 different cases of systemic lupus were tested in this manner. Two of the sera developed a faint precipitin line which was just visible at the end of the second week and gradually increased in intensity to the end of the third week (plate 11). The position of the line was found to be altered by reduction of the D.N.A. concentration to 0.25 mg/ml. when the precipitin band appeared nearer the D.N.A. agar interface.

Precipitin tests with histone

Capillary tube method:

Calf thymus histone (Worthington Biochemical Corporation) was used in these experiments. The basic protein histone is soluble in acid solutions and was dissolved in 0.5% citric acid, the final pH of the solution was 4.0. Concentration of 5 mg/ml., 2.5 mg/ml., 1 mg/ml., 500µg/ml. and 50µg/ml. histone were made up in the citric acid solution

and capillary tube precipitin tests were set up with normal and anti-nuclear factor positive sera. In the tubes containing 5 mg/ml. of histone, intense precipitation was found with all the sera within 15 minutes at 37°C. No precipitate had formed in this period at any of the other dilutions or in the control tubes containing serum and the citric acid diluent only. At 24 hours, slight precipitation was present in the tubes containing 2.5 and 1 mg/ml. of histone but no differences were found between the normal and anti-nuclear factor positive sera. The pH of the histone solution was raised to 6.0 by the addition of 0.2 M. triss acid maleate and 0.2 M. NaOH. This solution gave a heavy precipitate with all sera within 15 minutes. An attempt was made to stabilise the histone solution (10 mg/ml. in 0.5% citric acid) by the addition of an equal volume of 1 M. sodium sulphate. Tests set up at the various dilutions of histone used above gave the same results as in the initial experiment.

Immunodiffusion tests

Immunodiffusion tests were set up on cellulose acetate paper soaked in phosphate buffer pH 7.0 in the manner previously described. Histone 1 mg/ml. in 0.5% citric acid was placed on a marked area in the centre of the paper and 2 anti-nuclear factor positive and 2 normal sera placed in marked areas 1 cm. distant from the histone. Diffusion was allowed to take place under liquid paraffin for 48 hours at room temperature and the paper washed and stained in the manner described. Areas of precipitate formation were formed opposite both the normal and anti-nuclear factor positive sera.

It was concluded from these experiments that by virtue of its affinity for serum proteins histone was an unsuitable material for this type of test.

CHAPTER 3.

B. STUDIES ON THE NATURE OF THE 'ANTIGEN'

1. Absorption Studies

Twentyfour sera with a positive anti-nuclear factor test were selected for absorption studies with nuclear components (table 17).

Nucleoprotein absorption

The nucleoprotein preparation used was a calf thymus preparation in 1M. NaCl (see Chapter 1) containing 2.4 mg. nucleoprotein per ml. (1.2 mg. DNA, 1.2 mg. histone). The addition of one part of 1/5 serum to one part of 2.4 mg. per ml. nucleoprotein gave the equivalent of 12 mgs. of nucleoprotein per ml. of serum. The mixture was incubated at 37°C. for one hour and overnight at + 4°C. This resulted in the complete or partial removal of the nuclear staining properties of the majority of the sera when tested by the fluorescent antibody technique, as compared with controls diluted with saline (Plate12) (table 17a).

Further absorption of the incompletely absorbed and the unaffected sera was carried out using the equivalent of 36 mgs. nucleoprotein per ml. of serum. The results are shown on table 17b.

The specificity of this nucleoprotein absorption was investigated using sera from cases of thyroid disease which contained both antithyroid antibodies and anti-nuclear factor. The fluorescent antibody technique has been used to show two antithyroid antibodies (anti-colloid and anti-cytoplasmic) (Holborow et al. 1959) by using slight

modifications of the anti-nuclear factor test:- For the detection of colloid antibodies, sections were fixed before serum treatment in 100% alcohol for 15 minutes. To detect cytoplasmic antibodies, it was necessary to omit the fixation in 95% alcohol after serum treatment, normally carried out for the anti-nuclear factor test. Two sera containing each of the three factors (anticolloid, cytoplasmic, anti-nuclear) were incubated as described with sufficient nucleoprotein to absorb the anti-nuclear factor, and then tested on sections of thyroid in the two ways described above. In each case, the removal of anti-nuclear factor by the nucleoprotein did not affect the cytoplasmic or colloid antibodies, thus confirming the specificity of the nucleoprotein absorption for anti-nuclear factor.

D.N.A. absorption

A specimen of each of the sera was mixed with DNA solution (Worthington Biochemical Corp.) to give a concentration of 5 mg. DNA per ml. of serum. A saline control was set up for each serum. After incubation at 37°C. for 1 hour and overnight at 4°C., the specimens were tested for anti-nuclear factor. In all cases the DNA had no effect on the factor. Three of the sera were diluted so that nuclear staining could just be detected and then tested with four different preparations of DNA (2 from Dr. P Kent, Oxford; 1 from The Chester Beatty Institute, and the commercial preparation mentioned above). The anti-nuclear factor was unaffected in each case.

Histone absorption

The same twentyfour sera were treated with calf thymus histone, and at a concentration of 10 mgs. of histone per ml. of serum anti-nuclear activity was removed. However, this absorption was non-specific, as this same concentration of histone was shown to remove thyroid antibody from two thyroid diseases sera. In addition, the same quantity of another basic protein (protamine) was shown to have the same effect.

TABLE 17 A

Showing Absorption of anti-nuclear activity by Calf Thymus nucleoprotein

	No. tested	Result using 12mg/ml.
Systemic Lupus Erythematosus	7	3 completely absorbed 4 trace remaining
Rheumatoid Arthritis	6	All completely absorbed
Cirrhosis of the liver	5	3 completely absorbed 1 trace remaining 1 unaffected
Thyroid Disease	6	4 completely absorbed 2 unaffected

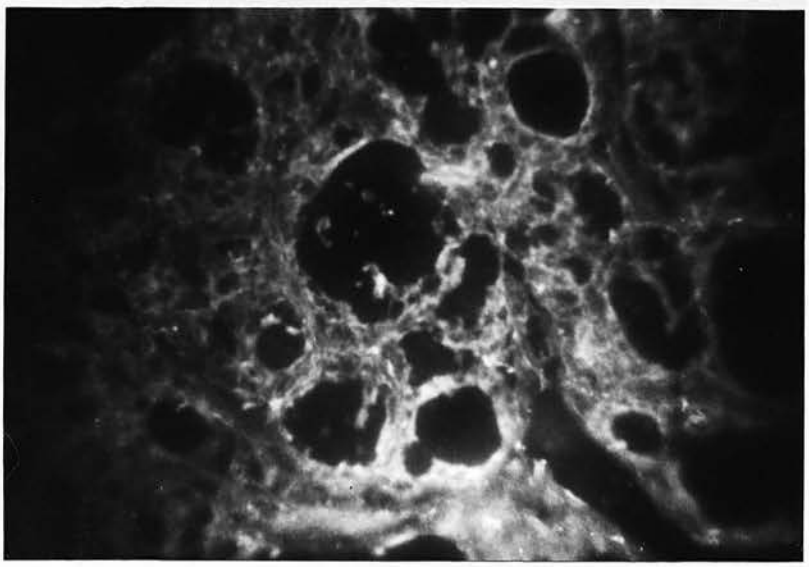
TABLE 17 B

Showing further absorption by Calf Thymus nucleoprotein of anti-nuclear activity from unaffected and incompletely affected sera of the above table

	No. tested	Result using 36mg/ml.
Systemic Lupus Erythematosus	4	no change
Cirrhosis of the liver	2	no change
Thyroid Disease	2	1 completely absorbed 1 unaffected

Plate 12.

Photomicrograph showing absence of nuclear staining of Thyroid cells following absorption of serum with calf thymus nucleoprotein.

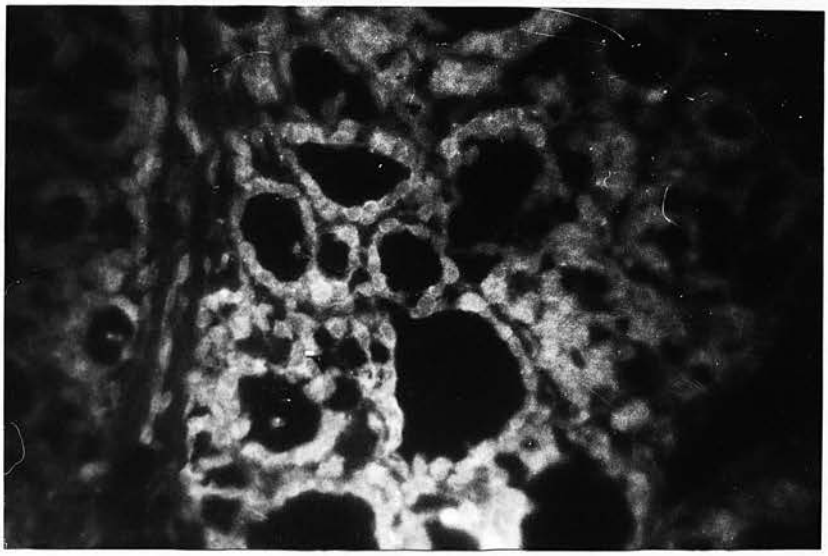


x 800

Note "holes" i.e. absence of fluorescence, in the position of the nuclei cf. plate 4.

Plate 13.

Photomicrograph showing result of D.N.A.'ase treatment of thyroid section following exposure to anti-nuclear factor.



x 800

Note persistence of nuclear staining (cf. plate 12).

2. Histochemical Studies

The study of the effect of various solvents and enzymes on the ability of nuclei to take up the anti-nuclear factor.

Nucleoprotein solvents

Nucleoprotein is soluble in either water or 1 molar NaCl.

Sections of infant thyroid were cut in the cryostat as already described for the anti-nuclear factor test and then layered with 1 M. NaCl or distilled water. After incubation with these solutions for 1 hour, the sections were washed in phosphate buffered saline pH 7.0 (Coon's saline) for 5 minutes, layered with anti-nuclear factor positive serum and then examined for the uptake of anti-nuclear factor in the usual manner. Apart from some blurring of the nuclear outline, nuclear staining was found to be present similar to that in control sections which had been exposed to normal saline instead of the nucleoprotein solvents. The failure to wash out the nucleoprotein from the cell nuclei was confirmed by the persistence of Feulgen staining of the nuclei. In view of the possibility that the viscous nature of nucleoprotein made its removal difficult, agitation of the sections in a bath of solvent was tried, but again this failed to achieve the desired removal of the nuclear material.

D.N.A'ase treatment of cell nuclei

(a) Before exposure to anti-nuclear factor:

D.N.A'ase (Worthington Biochemical Corp.) was used at a concentration of 1 mg/ml. in 0.003 Molar $Mg.SO_4$. It was found that

treatment of tissue sections with this solution for 1 hour at 37°C. was sufficient to remove the D.N.A. from the nuclei of tissue sections as judged by the absence of Feulgen positive material when the sections were stained by the Feulgen process. Sections treated with the $MgSO_4$ solution alone was unaffected.

The ability of nuclei of tissue sections treated with D.N.A'ase to take up anti-nuclear factor was tested with a number of anti-nuclear factor positive sera namely:- 10 sera from cases of systemic lupus, 2 sera from cases of cirrhosis of the liver, and one each of rheumatoid arthritis and thyroid disease. The liver and thyroid disease sera were chosen as it had not been possible to absorb the anti-nuclear factor activity of these sera by the addition of calf thymus nucleoprotein (table 17), and it was considered that these sera may be reacting with a component of the cell nucleus other than nucleoprotein. In all cases, however, no anti-nuclear factor was taken up by the D.N.A'ase treated cell nuclei.

(b) After exposure to anti-nuclear factor:

The effect of treating tissue sections with D.N.A'ase after they had been exposed to serum containing anti-nuclear factor was tried using the same 10 sera from patients with systemic lupus as in the previous experiment. The same conditions of D.N.A'ase treatment and washing were used and controls were included in which $MgSO_4$ solution was used instead of D.N.A'ase. The results showed that, despite the fact that as judged by the Feulgen stain the D.N.A. had been removed by the D.N.A'ase treatment, no change in the anti-nuclear factor staining was present with any of the sera. (plate 13).

Treatment of tissue sections with citric acid (to remove the basic proteins from the cell nuclei - Holman & Kunkel 1957).

(a) Before exposure to anti-nuclear factor:

Infant thyroid sections were exposed to citric acid for 30 minutes at 37°C. followed by 5 minutes wash in phosphate buffered saline (pH.7.0). The sections were then layered with three anti-nuclear factor positive sera from systemic lupus patients and with normal serum controls. When tested for the presence of nuclear staining by the fluorescent antibody method, none of the sera were found to react with the citric acid treated cell nuclei. The Feulgen stain was found to be unchanged by the citric acid treatment.

(b) After treatment with anti-nuclear factor:

In the same way as in the D.N.A.'ase experiment, treatment of serum treated tissue sections with 1% citric acid failed to affect the nuclear staining by anti-nuclear factor positive sera.

Treatment of tissue sections with D.N.A'ase and citric acid successively.

Serum treated tissue sections were exposed first to D.N.A'ase and then to citric acid under the same conditions as above. No change was found in the anti-nuclear staining when the sections were examined under the fluorescence microscope.

The effect of a number of other enzymes was tried on thyroid tissue sections as follows:-

Ficin 0.1% in N.sal; Papain 0.1% in a solution pH.6.0 containing the following:- Cysteine 0.605 gms.per litre, Na_2EDTA 1.86 gms.per litre, citric acid 7.74 gms.per litre, Na_2HPO_4 18.03 gms.per litre; Trypsin 0.01% and 0.001% in borate buffer pH 8.0; Pepsin 0.1% in N/10 HCl. Sections were treated with these enzymes for periods of 5, 10 and 30 minutes, and control sections with the solvents only at 37°C. These sections were then treated with three anti-nuclear factor positive SLE sera and normal sera controls, and tested for nuclear staining by the fluorescent test. The Feulgen staining properties of the nuclei were unchanged by this treatment. There was, however, a certain amount of swelling of the nuclei in the pepsin treated sections. None of the sera showed any alteration in their ability to stain the treated sections compared with the controls.

The use of guinea pig testis and smears of spermatazoa as a substrate for the anti-nuclear factor test

In view of the evidence of Vendrely et al. (1957) that mature sperms are deficient in histone, sperm preparations were used in an attempt to indicate whether histone is required for the uptake by nuclei of anti-nuclear factor.

Sections of fresh guinea pig testis were prepared in the same way as for infant thyroid. These were layered with 10 different anti-nuclear factor positive sera and tested for nuclear staining in the anti-nuclear factor test. When examined under the fluorescence microscope the somatic nuclei of the testis and epididymis fluoresced brightly, as did the immature germ cells in the peripheral parts of the follicles. However, the mature spermatozoa both in the germinal follicles of the testis and in the epididymis failed to show any staining (plate 14). Vendrely et al. (1957) considered that the developing germ cells gave up their nuclear histone just prior to maturation into adult spermatozoa and the evidence described here corroborates a change of nuclear constitution at this stage.

The possible presence of a physical barrier preventing the uptake of anti-nuclear factor by the sperm was investigated by the use of various enzymes and by chemical and physical methods as follows:-

(a) Enzymes:- Ficin, papain, trypsin and pepsin (made up in the same strengths and in the same solvent buffers as previously described) were layered on to sections of testis, left for 5, 15 and 30 minutes

at 37°C., and then washed off by buffered saline. Staining with anti-nuclear factor positive sera and with negative control sera showed that there was no difference in the appearance of the treated sections compared with that of the control untreated sections, and the spermatozoa remained unstained.

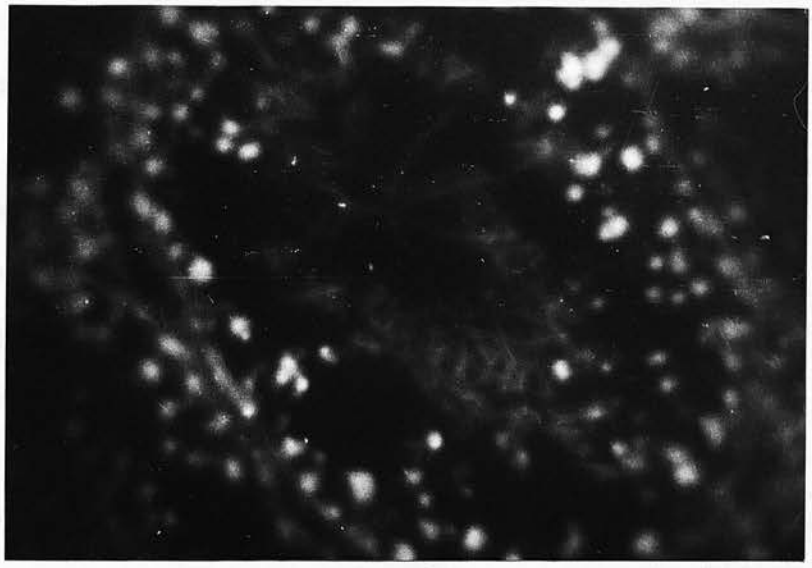
(b) Chemical methods:- The sperm cell membrane contains sulphur-containing amino acids and, to dissolve this keratin, thioglycollic acid (B.D.H)($CH_2SH.COOH$) was added to a suspension of guinea pig sperms (obtained by milking the epididymis taken from a freshly killed animal and collecting the spermatozoa in normal saline). Microscopic examination of a sperm suspension containing 10% thioglycollic acid showed on wet films that no motile sperms were present after 1 minute exposure to the acid. The mixture of spermatozoa and thioglycollic acid were incubated for a period of 4½ hours at 37°C. and specimens were removed at 30 minute intervals. Smears were then made on glass slides, dried in air, and layered with anti-nuclear factor positive sera and with normal control sera. Testing for nuclear staining in the usual way showed no nuclear staining of the sperm heads at any stage.

(c) Physical methods:- Guinea pig suspensions were exposed to high frequency sound waves in an M.S.E. Mullard cabinet at 20 kilocycles per second. After 5 minutes, microscopic examination of wet films showed an almost complete absence of motile cells and numerous free sperm heads and separated tails lying free. However, when tested with anti-nuclear factor positive sera, no uptake of the factor was observed, even by the free sperm heads.

Plate 14

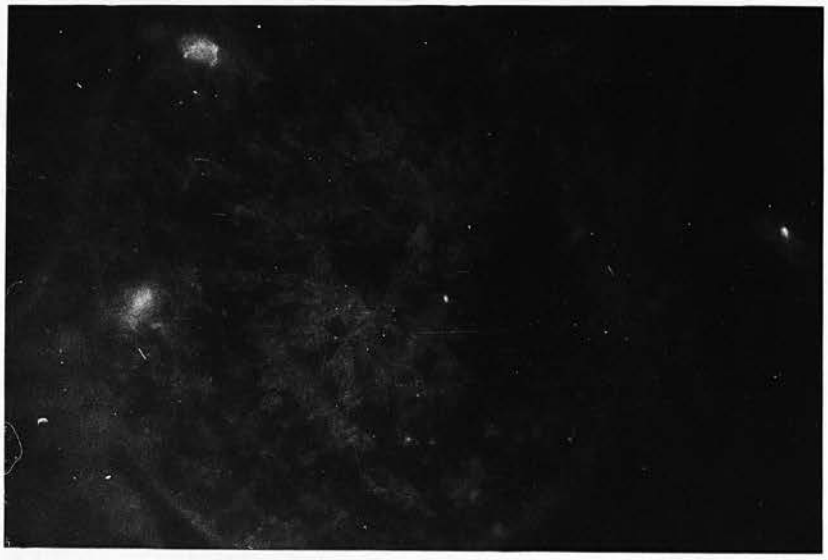
Photomicrographs of sections of Guinea-pig testis treated with anti-nuclear factor positive serum and normal serum.

treated with anti-nuclear factor positive serum



x 800

treated with normal serum



x 800

Note absence of staining of sperm heads; the slightly auto-fluorescent tails can be seen near the centre of the germinal follicle.
The somatic nuclei and the immature sperm cells take up anti-nuclear factor from the positive serum.

3. Immunisation with nuclear materials

Animal experiments were undertaken in an attempt to induce immune responses to various nuclear components. It was hoped that either circulating antibody (comparable to the anti-nuclear factor) or skin hypersensitivity reactions (of the immediate (within 4 hours) or delayed (24 hour) types), could be produced experimentally. Particular interest in the delayed hypersensitivity reaction arose from the accumulating evidence of the importance of cellular immunity in the pathogenesis of systemic lupus erythematosus.

The source of the materials mentioned in table 18 was as follows:- Calf thymus nucleoprotein and guinea pig nucleoprotein prepared as described in Chapter 1; Calf thymus D.N.A. and calf thymus histone from Worthington Biochemical Corporation; Polylysine, synthesised by Courtaulds for Dr. R. Consden; Bovine serum, albumin and fraction 2 gamma globulin from Armour Laboratories; protamine sulphate from British Drug Houses. Finally the calf thymus saline extract was taken during preparation of nucleoprotein, and consisted of the supernatant obtained after the precipitation of nucleoprotein by 6 volumes of water from the 1 M. saline solution (see Materials and Methods Chapter). The protein content of this solution was 2 mg/ml. (Biuret).

Immunisation was carried out by the subcutaneous or intra-dermal injection of the materials with or without adjuvant (Freund's complete or incomplete - Difco).

Guinea Pigs

Forty-one animals received one injection each of calf thymus nucleoprotein in 1 M. saline, the amount ranging from 4 µg to 2.5 mg.

Twelve animals received 600 µg. of nucleoprotein with complete adjuvant intradermally at weekly intervals for 3 weeks. Skin tests in these animals 7 days later using 1/5 or 1/20 dilutions of the test antigen injected intradermally in quantities of 0.1 ml. gave rise to delayed skin sensitivity to the nucleoprotein, whether or not adjuvant was used. However, skin sensitivity, both immediate and delayed, and anaphylaxis, were elicited also by a saline extract of calf thymus, and in addition delayed skin reactions occurred to bovine serum, bovine albumin and fraction 2 gamma globulin. In contrast, no reactions were obtained to calf thymus D.N.A. or to guinea pig nucleoprotein.

The conclusion was drawn that the above reactions could be the result of immune reactions to contaminating material in the nucleoprotein preparation. Accordingly, this was tested by immunising animals with saline extract of calf thymus and complete adjuvant, and with a complex of calf thymus D.N.A. and bovine albumin. Animals immunised in this way showed delayed reactions to calf thymus nucleoprotein, to saline extract of calf thymus, to calf thymus histone and also to bovine albumin. The animals showed no reaction to D.N.A. These results, therefore, could all be attributed to delayed reactions, involving bovine serum albumin present in the preparations of nucleoprotein used for immunisation and in the reagents used for skin testing.

Table 18

SKIN TESTS AND ANAPHYLAXIS IN GUINEA PIGS IMMUNIZED WITH NUCLEAR MATERIALS

Immunizing Material and Doze	No. of Guinea Pigs & Route	Skin Tests Immediate	Skin Tests Delayed	Testing Material	Anaphylaxis
Calf thymus nucleoprotein (NP)		0	+	Calf thymus NP	0
(in 1M sal.4 µg-2.5mg.)	* 38 i.d.	(+)	++	Saline Extract of calf thymus	++(death)
with complete or incomplete		(+)	++	Bovine serum, FII and albumin	-
adjuvant or without adjuvant	15 s.c.	0	0	DNA (Calf thymus)	-
		0	+	Histone (calf thymus)	-
		0	0	Guinea pig NP	
Saline extract of calf		0	(+)	Calf thymus NP	0
thymus (2 mg. protein)	4 i.d.			Saline Extract of calf	
(complete adjuvant)		(+)	(+)	thymus	++(death)
Insoluble complex from:-				Calf thymus NP	
DNA (300 µg) + Histone(300µg.)	2 i.d.	0	0	DNA	
ditto. + Protamine(125µg.)	2 i.d.	0	0		-
DNA (500 µg) + Polylysine (2.5mg)	2 i.d.	0	0	Histone, protamine, polylysine	
(in complete adjuvant)					
Calf thymus DNA		0	+	Calf thymus NP	
Complex with bovine albumin	2 i.d.	0	+	Saline extract calf thymus	
(125 µg DNA+2.5mg.BSA)		0	0	DNA	-
(complete adjuvant)		0	++	Bovine albumin	
Calf thymus Histone (500µg)		0	0	Calf thymus NP	
Polylysine 2.5mg, or		0	0	DNA	-
Protamine (125mg)	6 i.d.	0	0	Polylysine, protamine, histone	
(incomplete adjuvant)					

* 12 animals of this group received 600µg. of nucleoprotein at weekly intervals with complete adjuvant for 3 weeks. Skin tests were carried out using 0.1 ml. of 1/5 or 1/20 dilutions.

Note: - = not done. i.d. = intradermal. s.c. = subcutaneous.

++ indicates raised erythematosis area 10-15mm. in diameter.
\+ ditto. 5-10mm. "
(+) ditto. 2- 5mm. "
0 indicates no reaction.

When such contaminants are not present as in artificially prepared complexes of "purified" D.N.A. and histone, no reactions are obtained following injections of this material. Injections of similar complexes with other basic proteins (protamine and polylysine) did not result in the development of skin reactions.

In addition to the skin tests, the sera (obtained by heart puncture) from 10 guinea pigs which had been immunised against calf thymus nucleoprotein, and which had shown positive skin reactions to this antigen, were tested for anti-nuclear activity by the indirect fluorescent antibody method on air dried and acetone fixed sections of calf thymus cut in the cryostat in the usual way. The fluorescent conjugate was prepared using an antiserum against guinea pig globulin prepared by the method of Milgrom et al.(1956) as described. Four other sera obtained from animals immunised against a complex of D.N.A. and histone were found to be negative.

Rabbit experiments

A number of rabbits were injected with nuclear materials as follows:-

Rabbits 1 and 2 were given 5 mg. of calf thymus nucleoprotein with Freund's complete adjuvant; rabbits 3 and 4 received 5 mg. of calf thymus histone with complete adjuvant; and rabbits 5 and 6 were given 5 mg. of calf thymus histone without adjuvant. The injections were given subcutaneously and repeated after 3 weeks. Ten days after the final injection, the animals were bled from the marginal ear vein and the serum collected. The sera from these animals was tested against sections of calf thymus and against human infant thyroid as

used in the anti-nuclear factor test. The fluorescent conjugate used was an antiserum prepared against rabbit globulin by extensive immunisation of a goat, using Freund's complete adjuvant (the serum was kindly provided by Dr. J. David). Examination of the preparations showed no evidence of nuclear staining with any of the sera. An interesting finding with the two sera from rabbits immunised against calf thymus nucleoprotein was weak cytoplasmic staining of the calf thymus cells. This staining would appear to be due to antibody to the contaminating protein material in the nucleoprotein preparation used for immunisation, and is consistent with the interpretation given to the results in the previous section.

Precipitin tests were carried out in capillary tubes with these sera, using calf thymus nucleoprotein, guinea pig nucleoprotein, calf thymus D.N.A., and bovine serum as the antigens. No precipitates were formed specific for the nucleoprotein preparations or the D.N.A. after 2 hours incubation at 37°C. and 72 hours at + 4°C. However, the sera from rabbits one and two showed a faint precipitate with bovine serum antigen at 72 hours.

C. Examination of tissues of patients with circulating anti-nuclear factor

During the course of this investigation, the tissues of 2 patients with circulating anti-nuclear factor became available.

1) Post-mortem specimens of heart, kidney and spleen from a patient with systemic lupus.
2) Spleen taken at operation from a patient with Rheumatoid Arthritis and splenomegaly.

Blocks of these tissues were snap frozen in the manner previously described and 6μ sections cut in the cryostat. The sections were layered with anti-human globulin fluorescein conjugate and examined microscopically for the presence of localised globulin. The following conclusions were made:-

(a) The nuclei of all the tissues examined showed no evidence of the in vivo uptake of serum anti-nuclear factor (plate 15).

(b) The nuclei of tissue sections could be coated by anti-nuclear factor from the patient's own serum when layered on in vitro (plate 15).

(c) Calf thymus nucleoprotein was conjugated with fluorescein isothiocyanate in the way described in Chapter 1 for protein conjugation (the protein concentration in this case was lower (1.2 mg./ml.) than that usually used for conjugation). When sections of spleen, either air dried in the usual way or fixed in anhydrous acetone, were layered with the conjugated nucleoprotein, no staining was noted by the cytoplasm or nuclei of any cells in the sections. The addition of unconjugated

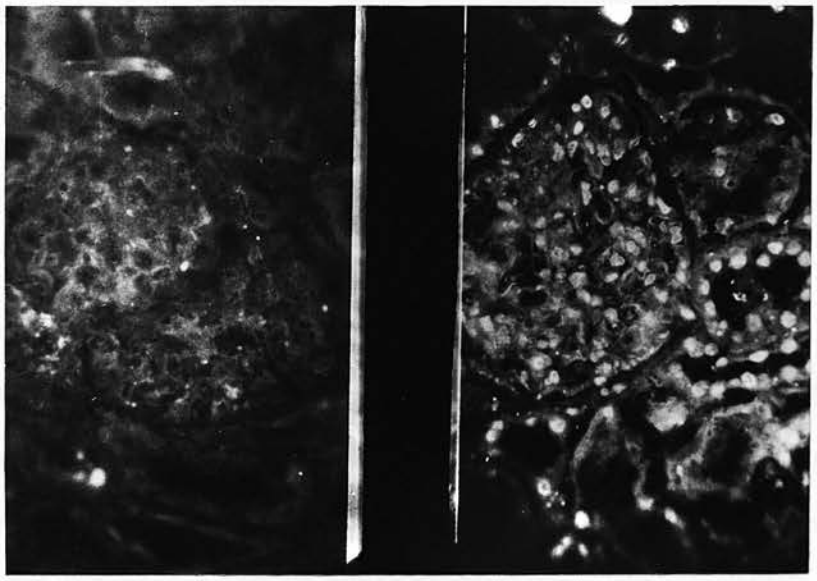
nucleoprotein followed by anti-nuclear factor positive serum and then by anti-human globulin conjugate also gave negative results.

(d) There was no evidence of uptake of gamma globulin by the glomerular tissue (plate 15). This did not, however, show any pathological change histologically.

It was not possible to show the specific localisation of gamma globulin in this kidney as the degree of background fluorescence present (plate 15) was similar to that in normal kidney, and the author would agree with Cruickshank's similar observations (1959) - see Introduction - who states that "it is difficult to see how this approach could be used to investigate the role of tissue auto-antibody."

Plate 15

Failure of uptake of anti-nuclear factor in vivo.



x600

Section of kidney from patient with S.L.E. stained with anti-human globulin conjugate, showing no nuclear staining.

Kidney section after exposure in vitro to same patient's serum and stained with anti-human globulin conjugate, showing nuclear staining.

DISCUSSION

This work has shown that using Coon's indirect fluorescent antibody technique it is possible to detect the coating of cell nuclei in unfixed tissue sections by a globulin factor derived from the serum of patients with Systemic Lupus Erythematosus and various other diseases (Table 1).

Comparison of Series

Systemic Lupus Erythematosus

The results have shown that this anti-nuclear factor is present in all cases of clinically definite Systemic Lupus Erythematosus except one in prolonged remission (described in the text). Fifty-eight of the 63 cases were shown to have a positive L.E. cell test. This high incidence of the anti-nuclear factor in Systemic Lupus has been found by other workers (Table 19). Thus Friou et al. (1958) using the same technique reported a series of 28 cases (active and quiescent) with a positive test in all but one, and in a later publication, using spots of dried calf thymus nucleoprotein instead of tissue sections, they obtained positive tests in all of 35 cases of the disease. Bardiwal et al. (1958) reported nuclear staining of tissue sections in all of 5 cases using both the indirect and the direct fluorescent antibody technique, and all fifteen of the Systemic Lupus cases of Calbresi et al. (1959) were positive for anti-nuclear factor using the indirect technique. Alexander and Duthie, using isologous white cells as the nuclear source, reported 8 cases with positive tests (1958), and later

Alexander, Duthie and Bremner reported 12 of 12 patients to be positive (1960). Mandema et al. (1961) tested 151 samples of sera from 56 patients and obtained 131 positive tests using buccal mucosal cells as the nuclear source. The 20 negatives were found in the "inactive stage" of the disease. Other recent reports using the same technique follow the same pattern - 35 positives among 39 cases (Baugh et al. 1960) - 22 of 22 cases (Hall et al. 1960) - 43 out of 53 cases (Rothfield et.al 1961) - 43 of 51 cases (Widelock et al. 1961).

Rheumatoid Arthritis

With regard to the incidence of anti-nuclear factor in Rheumatoid Arthritis, the finding in the present series that 19 of 132 cases were positive (14%) compares well with the findings of Friou et al. of 3 positives among 15 cases using tissue sections and 4 positives of 42 cases using nucleoprotein spots, and that of Mandema et al. (1961) of 2 positives in 17 cases. Two positives among 11 "uncomplicated" cases of rheumatoid arthritis were reported by Calbresi et al. (1959): only 3 of 39 cases had anti-nuclear factor titres of over one in eight in the 9 positives found in the series of Baugh et al. (1960): 5 positives were found in 25 cases of Rothfield et al. (1961). In contrast, Bardiwal et al. (1958) found 5 of 8 cases of Rheumatoid Arthritis to be positive, but as 4 of the 5 cases had a positive L.E. cell test compared to the finding in the present series of only 3 positives in 95 cases, their

series would appear to be non-representative. The same workers (Hall, Bardiwal, Bayles and Mednis 1960) reported on 130 cases of rheumatoid arthritis and found anti-nuclear factor in 36% of the cases. L.E. cells were found in 13% of the cases. Alexander et al. (1960) reported a high incidence (65%) in a large series of 183 patients with Rheumatoid Arthritis. However, if we examine the titres reported by Alexander in both Systemic Lupus Erythematosus and Rheumatoid Arthritis, and compare them with those in the present series, we see a striking difference in the sensitivity of the tests used. Eight of the 14 cases of Systemic Lupus titrated in the present series had titres of between 1/100 and 1/300 and none exceed this titre (the test in this respect compares closely to that of Friou et al.). By contrast, however, Alexander et al. report that 5 of their positive sera could be diluted beyond 1/512, and one of these to 1/4000. Similarly, the titres of anti-nuclear factor in sera from Rheumatoid Arthritis patients in the present series, with two exceptions, fall below 1/100 in the 12 positive cases tested (again following a pattern similar to that of the cases of Friou et al.). In comparison, Alexander et al. reported that 14 of the 119 anti-nuclear factor positive cases in their Rheumatoid Arthritis series had titres of 1/512 and 6 at dilutions even greater than this.

TABLE 19

Showing Incidence of anti-nuclear factor in Systemic Lupus and Rheumatoid Arthritis using the fluorescent antibody technique

	Systemic Lupus		Rheumatoid Arthritis	
	positive	negative	positive	negative
Present series	62	1	19	113
Friou et al.	27	1	3	12
Bardiwal et al.	5	5	5	8
Calbresi et al.	15	0	2	11
Alexander et al.	12	0	119	44
Baugh et al.	35	4	9	30
Hall et al.	22	0	46	84
Goodman et al.	26	1	6	24
Mandema et al.	131(sera)	20	2	15
Rothfield et al.	43	10	5	20
Widelock et al.	43	8	16	32

Other Diseases

The finding of anti-nuclear factor in 2 of 4 cases of Dermatomyositis in the present series is consistent with similar reports by Friou et al. (1958) - one of 2 cases positive, Hall et al. (1960) - 2 out of 3 cases positive, and Alexander et al. (1960) - one case tested and found positive. The failure to find anti-nuclear factor in the 8 cases of Scleroderma tested contrasts with the finding of 1 among 3 cases reported by Friou et al. (1958), 2 of 3 cases by Alexander et al.(1960), 4 of 6 by Bardiwal et al. (1958), and 8 of 10 cases by Hall et al.(1960). However, until a larger series is available, it is not possible to do more than state the difference in incidence which has been noted. The finding in the present series of positive tests in sera from patients with liver disease was confirmed by Mandema et al.(1961) who reported the finding of 5 positive tests in 11 cases of hypergammaglobulinaemia and liver disease, and by Baugh et al. (1960) who found 3 cases with a positive test in eight cases of chronic liver disease. There are no other reports of positive anti-nuclear factor tests in Stills disease (in the present series 13 of 100 cases were positive); the small number of cases (7) tested in the reported study of Alexander et al. (1960), all of which were negative, does not allow a final conclusion to be drawn. Similarly, no comparable series of Discoid Lupus patients has been tested by this technique. The series reported by Hijmans et al. (1961) give an incidence of 8% in their 182 cases of uncomplicated Hashimoto's disease compared with 14% in the present series. White et al. (1961) described anti-nuclear factor in 5 (12%) of 40 cases of lymphadenoid goitre.

Comment on the clinical significance of anti-nuclear positive reactions

Systemic Lupus Erythematosus

The series of 63 cases of Systemic Lupus reported in this work shows a 98% incidence of anti-nuclear factor in the disease. As shown in the previous section, all the reported series of cases of this disease give a high incidence of the factor. In addition, Table 3 shows that all the Systemic Lupus cases fell into the moderate or high titre groups and none had a titre under 1/20. This is consistent with the findings of Mandema et al. (1961) and Baugh et al. (1960). Thus it would be difficult to sustain a firm diagnosis of Systemic Lupus in the absence of a positive test for anti-nuclear factor. A positive result would be of particular value in cases in which the L.E. cell test had been found to be negative, as applied to 4 of the cases in this series (and 15 of the 35 anti-nuclear factor positive cases of Baugh et al. 1960). The author would strongly concur with the view that "the diagnostic value of anti-nuclear reactions in this condition is beyond question" (Nairn 1962). It is important to stress that the results are reliable only if proper controls are carried out at each investigation. In the author's experience, the anti-nuclear factor test is much simpler to interpret than the L.E. cell test, and individual tests can be more rapidly read. Tables 4, 5 and 6 show the incidence of certain features in the present series and in other reported series which are of interest in relation to possible

immunological abnormalities in the disease. Whilst each feature is capable of an alternative explanation, their occurrence together is consistent with the outline in the introduction in relation to possible occurrence of auto-antibodies to red cells (anaemia and positive Coombs test), to white cells (leucopenia), platelets (thrombocytopenia) and anti-tissue antibodies (positive serological tests for syphilis). The immunological evidence to be discussed hopes to show that anti-nuclear factor can be included with these as a possible auto-antibody.

Rheumatoid Arthritis

In their study of the incidence of anti-nuclear factor in human serum, Alexander et al. (1960) used a sensitive technique which resulted in the detection of anti-nuclear factor in 65% of 183 cases of rheumatoid arthritis. This resulted in a large series of anti-nuclear factor positive cases of rheumatoid arthritis and they were able to present a comprehensive analysis of their patients on a comparable age and sex basis, emphasising primarily the importance of anti-nuclear factor in relation to prognosis. An association was found in their series, of anaemia (haemoglobulin of less than 70%) and a raised E.S.R. with the presence of anti-nuclear factor; they also noted that long standing cases with impaired functional capacity tended to fall into the anti-nuclear factor positive group. They came to the conclusion that "The results of the present study confirm that both anti-nuclear factor and rheumatoid factor may be of prognostic significance and that

anti-nuclear factor was more closely associated with signs of the disease and with functional activity". The present series, in comparison, included a much smaller number of anti-nuclear factor positive cases in the total of 132 cases of Rheumatoid Arthritis tested, and did not allow an analysis of this type. A comparison between anti-nuclear factor positive and negative cases was made, however, with emphasis again on features relevant to the auto-immune hypothesis and a possible underlying relationship between Rheumatoid Arthritis and Systemic Lupus. The important point of agreement between the series of Alexander et al. and the present one was the significance of anaemia in relation to the presence of anti-nuclear factor. In the present series a X^2 of 6.71 was found when comparing the occurrence of anaemia (Hb. less than 11g.) in the anti-nuclear factor positive group with its incidence in the negative group. There was no significant difference between the globulin levels (table 7) in the anti-nuclear factor positive and negative groups of cases, and thus if any difference did exist it must have been qualitative rather than quantitative. The presence of the rheumatoid factor, which indicates a qualitative change in the gamma globulin of these patients, was not significantly different between the two groups, this factor being a common feature to the majority of Rheumatoid cases. A feature noted in the present series and not included in the analysis of Alexander et al. (1960) was L.E. cells, which as might be expected were found in a significant proportion (25%)

of the anti-nuclear factor positive cases. The comparison in table 8 suggests that the Rheumatoid Arthritis cases, with respect to the features listed, resembled Systemic Lupus more closely when anti-nuclear factor was present in their serum than when it was not.

Discoid Lupus

The 75 cases in this group, as has been stated, were carefully selected because extensive study had shown no evidence of systemic disease at all - and although the extent and the severity of the skin lesions varied (and indeed corresponded to the presence of anti-nuclear factor)all were following a long benign course. This makes the presence of anti-nuclear factor in 13% of the cases a surprising finding and one of considerable interest in relation to the question of the overlap of this disease with systemic lupus (Scott and Rees 1960). Up to 6 months after the tests were carried out, the anti-nuclear factor positive cases are reported not to have developed any systemic signs (Scott personal communication). There is no other comparable series reporting tests for anti-nuclear factor in this condition. However, the report of Bennett et al. (1961) does offer some points of comparison. These workers studied 16 cases of Discoid Lupus which at the outset of the investigation had no evidence of systemic disease. The tests carried out included a complement fixation test between D.N.A. and patient's serum (uniformly negative), the L.E. cell test (positive in 2 cases one of whom later developed signs of systemic disease), a skin test

using leucocytes - this test having been shown to be positive in a significant proportion of cases with the systemic disease (3 of the 16 cases gave a positive reaction). The indirect Coombs test for rheumatoid factor and the Wassermann were positive in a few of the cases. These results taken with the present findings emphasise the probable underlying relationship between the purely cutaneous and the systemic forms of the disease.

Thyroid Disease

The significance of anti-nuclear factor in thyroid disease is probably most clearly summed up in the report of Hijmans et al.(1961) who have carried out an extensive study of auto-antibodies in thyroid disease, systemic lupus, rheumatoid arthritis and various combinations of these diseases. They state "The two diseases lymphadenoid goitre and systemic lupus appear to be extreme examples at opposite ends of a spectrum of diseases showing varying immunological disturbances." In support of this thesis they show that 15 (25%) of the 60 systemic lupus cases have anti-thyroid antibodies, that all 5 of the cases with co-existent Hashimoto's disease and systemic lupus have both anti-nuclear factor and anti-thyroid antibodies, and that 8% of their uncomplicated Hashimoto's disease cases have anti-nuclear factor. In their cases of Rheumatoid Arthritis 9 (11%) of the 79 cases uncomplicated by thyroid disease had anti-thyroid antibodies and all 7 of the cases in which

both diseases coexisted had anti-thyroid antibodies and three had anti-nuclear factor. It is of special relevance to the present study that anti-nuclear factor should co-exist with thyroid antibodies in the well-established auto-immune condition of Hashimoto's disease. (see page 164).

Liver Disease

The anti-nuclear factor has not been widely studied in liver disease as already noted. The findings in the present series were in agreement with those of Baugh et al. (1960) with respect to the titre of the factor, which fell into the moderate titre category in both series. The L.E. cell test, on the other hand, has been examined for, and found to be positive in cases of disease in several reports. Heller et al. 1956 reported 3 cases of post-necrotic cirrhosis with a positive L.E. cell test, Joske and King in 1955 reported 2 cases of chronic active hepatitis with L.E. cells, and one case was found in the report of Wilkinson and Sacker (1957). The detailed report of 14 cases of liver disease with L.E. cell factor by Mackay et al. (1959) used the term "Lupoid hepatitis" to describe the condition, and it is of interest to compare their L.E. cell positive sera with the anti-nuclear factor positive cases in the present series. In their series, 6 of the cases had arthralgia as one of the symptoms, and in the present series 3 of the 5 positive cases had this feature compared with only 2 of the 22 negative cases. In addition, two of the 3 cases with arthralgia and anti-nuclear

factor had a positive test for rheumatoid factor. (Table 10). Four of the five anti-nuclear factor positive cases in the present series were considered to be post-infective forms of the disease and this consideration applied to 5 of the 14 cases in the series of Mackay et al. (1959). (7 other of their cases had a doubtful aetiology). Whilst 10 of their cases were females, only 2 of the 5 cases in the present series were females. One case in each series had haemolytic anaemia. Mackay et al. noted in liver biopsies of 9 of their patients that dense plasma-cell and lymphocytic foci were prominent in the portal tracts as "the morphological counterpart of antibody production" In addition, the auto-immune complement fixation test (A.I.C.F) was found to be positive in 75% of their cases, The histological findings in the livers of 19 cases of systemic lupus studied by Mackay and his colleagues showed non-specific changes in 13, and normal tissue in four. The remaining two had the typical "Lupoid hepatitis" changes. These findings are of interest in view of the overlap which has been shown between the Lupoid hepatitis cases and the present series of anti-nuclear positive cases.

Consideration of the Low-Titre Anti-Nuclear Factor Positive Sera

Occasional low-titre positive tests were found in the present investigation in patients with miscellaneous disease, in the pulmonary tuberculosis group and in the normal controls. Titration of these sera has shown that they fall into the low titre category. There are a number of possible interpretations to account for these findings. The first is to assume that the anti-nuclear factor in all these sera is the same as that in Systemic Lupus and that the difference is quantitative. In this case either the anti-nuclear factor is present in all normal sera in small amounts and is enhanced in certain diseases (discussed Page126) or the finding of anti-nuclear factor is a reflection of underlying disease which will emerge at a future date. An alternative hypothesis is that the anti-nuclear test is able to detect a number of different serum components which can react with cell nuclei, some of these reactions being non-specific. For example, the factor in Systemic Lupus may be a true auto-antibody and that in Rheumatoid Arthritis and in other conditions some other factor such as lysosyme (Potter et al. 1961), or a reaction between serum albumin and D.N.A. of the type described by Goldwasser and Putnam (1956). The finding of anti-nuclear factor in some relatives of patients with Systemic Lupus reported by the author with Ansell and Johnson (1961) and by Mandema et al.(1961) suggests the possibility that this may be an early reflection of the disease, and is supported by the well described

familial incidence of the disease (see Discussion p.167). Thus the finding of anti-nuclear factor in a small proportion of the control series may reflect a predisposition to abnormal antibody production and an indication that such individuals are candidates for auto-immune disease.

The possibility that the anti-nuclear factor test may be able to detect a number of different non-antibody serum components capable of reacting with cell nuclei must be examined closely. Potter, Duthie and Alexander (1962) found that a variety of proteolytic enzymes, when conjugated with fluorescein, combine with cell nuclei and give typical anti-nuclear staining (i.e. using the direct method of Coons). Since the sera of patients with Rheumatoid Arthritis were able to bind smaller quantities of papain than normal sera, these workers postulated that enzyme present in such sera may be unbound and free to produce some of the lesions noted in the disease. They also suggested that such enzymes may be responsible for nuclear staining at least in some cases. To support this thesis they found that the bacteriolytic (lysosyme) activity of the serum was increased in most Rheumatoid Arthritis patients who had anti-nuclear factor. They did not, however, find this association in Systemic Lupus. They did not absorb the enzyme from the sera and then test to see if anti-nuclear activity remained following this. There are several facts which make it unlikely that enzymes are responsible for the staining described in the present work. Papain and a number of other proteolytic enzymes were used in the

present work for treating cell nuclei (page 102) and in the control tests no nuclear fluorescence was observed. Similarly, papain-treated human sperms provided by Dr. Lachmann did not take up fluorescent anti-human globulin conjugate. Thus it appears that the conjugates used in the present work would be unlikely to detect such enzymes, since such detection would rest on the specificity of the particular fluorescent conjugate used(in the indirect method of Coons). According to Potter et al. (1962) free enzyme moves with the gamma globulin fraction of serum on electrophoresis. The fact that conjugate No. 1 (plate 5) gives only one main precipitin band with the gamma globulins, and that there is no report of precipitin bands obtained against lysosyme in human serum (see Crowle 1961) in immunoelectrophoretic studies using a large variety of anti-human sera, makes it unlikely that such a conjugate would detect the enzyme on cell nuclei.

Finally, Potter et al. do not report any immunodiffusion precipitin tests or attempts to absorb antibody to lysosyme from their conjugates, to support their suggestion that their conjugate was able to detect this enzyme. The remaining possibility that the reactions were of a non-immunological type due to the reaction between D.N.A. and serum albumin is considered later in the discussion (page 154).

Detection of reactions to nuclear material by other immunological techniques

1) The antiglobulin consumption test - in which the titre of anti-human globulin serum is measured after the addition of cell nuclei which have been exposed to test serum. The results obtained using this method have been tabulated (Table 20), and show a sensitivity closely comparable both as regards Systemic Lupus and Rheumatoid Arthritis with that of the fluorescent antibody technique as reported in the present work. In addition, Miescher et al.(1957) report positive tests in "other connective tissue diseases" (not included in the table) without giving detailed incidences.

2) The use of radioactive isotope labelled anti-human globulin to detect the uptake of anti-nuclear factor on cell nuclei was described by Friou (1958) employing I^{131} to study a small number of cases. With 4 Systemic Erythematosus sera, counts of 550-4000 per minute were observed, compared with 50-60 per minute in 2 sera from Rheumatoid Arthritis patients, and counts of 5, 3 and 9 in the three normal sera tested. The author suggested that the technique might be a valuable method of quantitating anti-nuclear factor in different sera, but there have been no further reports using this technique.

3) Passive haemagglutination tests in which sheep or human red cells are treated with dilute tannic acid, coated with nuclear material and then agglutinated by sera containing antibody to this nuclear material, have

been used by several workers (see table). Not all cases of Systemic Lupus Erythematosus are positive in this test (see Table 20), and no positives are reported in sera from cases of Rheumatoid Arthritis, except for 3 cases in the series of Goodman et al. (1960).

4) The inert particles polystyrene, latex and bentonite coated with calf thymus nucleoprotein (Christian et al. 1958; Fessel 1959) or Calf thymus D.N.A. (Bozicevich et al. 1960) have been used to test sera for activity against nuclear material. Like the tanned cell test, a substantial number of Systemic Lupus Erythematosus sera were negative in this test. Fessel found that four of these negative sera which did not agglutinate the particles were L.E. cell positive. Whilst Christian et al. and Bozicevich et al. report no positive results in Rheumatoid Arthritis, Fessel found an incidence of 22% in his series of 150 cases. The latter also reported several positives in sera from patients with a positive Wassermann test, some of whom had definite clinical evidence of syphilis. Using a commercial latex test (Hyland L.E. test), Dubois et al. (1961) found only 24 positive of 154 cases of Systemic Lupus compared with 79 positive L.E. cell tests.

5) Complement fixation tests have been used for the detection of antibody-antigen combination, using whole nuclei, calf thymus nucleoprotein, D.N.A. and nucleoprotein as the antigens. There is considerable variation in the incidence reported in Systemic Lupus

Erythematosus, and to a less extent in Rheumatoid Arthritis, which cannot be related to the nuclear component used. Thus Robbins et al. (1957) found that 8 of 9 L.E. cell positive sera from patients with Systemic Lupus fixed complement in the presence of calf thymus nucleoprotein. Using the same antigen Rothfield et al. (1961) obtained 23 positive tests in 35 cases of the disease with the very sensitive conglutinating complement fixation test. Using D.N.A. as the antigen, Pearson et al. (1957) and Seligmann (1959) obtained an incidence of 35% and 57% respectively. In contrast, Asherson (1959) found only 4 positive sera in 25 cases of Systemic Lupus which fixed complement in the presence of rat liver nuclei, calf thymus nucleoprotein and D.N.A. The comprehensive study of Hijmans and Schuit(1959) using sera from 29 cases of the disease found that 16 sera fixed complement in the presence of whole nuclei, and that 12 of these did so with calf thymus nucleoprotein, 6 of these with D.N.A., and 2 with histone. Hijmans and Schuit also studied sera from patients suffering from Rheumatoid Arthritis and obtained 7 positive tests with whole nuclei, 4 with nucleoprotein and one each with D.N.A. and histone. Two of the 37 Rheumatoid Arthritis cases of Rothfield et al. fixed complement with nucleoprotein and D.N.A., and one case reacted with histone.

6) Precipitation reactions between Systemic Lupus Erythematosus sera and D.N.A. and nucleoprotein have been used in the present series and by

TABLE 20

Showing Incidence of anti-nuclear factor in Systemic Lupus and Rheumatoid Arthritis. Comparison of series using techniques other than fluorescent antibody technique.

1. Antiglobulin consumption test

	Systemic Lupus		Rheumatoid Arthritis	
	positive	negative	positive	negative
Miescher et al.	17	0	10	41
Engelfriet et al.	28	0	-	-

2. Tanned cells and inert particles

Miescher et al.	3	2	0	25
Epstein & Lee	10	0	0	10
Christian et al.	19	5	0	53
Fessel	25	12	33	117
Bozicevich et al.	10	3	0	6
Goodman et al.	23	4	3	27
Dubois et al.	24	130	-	-

/continued

TABLE 20 (contd.)

Showing Incidence of anti-nuclear factor in Systemic Lupus and Rheumatoid Arthritis. Comparison of series using techniques other than fluorescent antibody technique.

		Systemic Lupus		Rheumatoid Arthritis	
		positive	negative	positive	negative
3. Complement fixation tests					
Robbins et al.		8	1	0	21
Pearson et al.		7	13	0	17
Hijmans et al.		16	13	7	32
Asherson		4	21	-	-
Seligmann		27	20	0	27
Rothfield et al.		23	12	2	37
4. Precipitin tests with D.N.A. or Nucleoprotein	(NP.)				
Present series	NP.	9	6	1	10
	DNA.	2	18	-	-
Polli et al.	DNA.	3	5	-	-
Klein et al.	DNA.	1	29	-	-
Seligmann	DNA	28	21	0	27
5. Precipitin and complement fixation tests with D.N.A.					
Seligmann		48	73	1	65 (incl. 24 L.E. cell positive)

other workers. Polli et al. (1957) used D.N.A. and obtained a precipitate in 3 of 8 cases, and Seligmann, also using D.N.A., obtained a precipitate in more than half his 49 cases. All the sera which showed precipitation were from untreated cases, and the sera from patients who had received treatment were usually negative. This explains the low incidence of precipitation with D.N.A. in the present series (2 of 20 cases); treatment would not appear to have this effect in the case of precipitation with nucleoprotein (see table 20). In a recent report, Seligmann (1961) reports the results of a study using both complement fixation and precipitation. Forty-eight of his 128 Systemic Lupus sera were positive (taking the result of both tests into consideration) and of these 26 of 29 untreated cases were positive and none of the 22 cases in remission.

Critical Evaluation of Techniques

The various techniques described above each have their own advantages and disadvantages.

The methods involving the identification of gamma globulin on cell nuclei appear to be the most sensitive way of detecting anti-nuclear activity in sera and of the three methods available - antiglobulin consumption test, isotope labelled antibody, and the indirect fluorescent antibody tests - the last is the only technique which has been applied to sufficient number of sera from different groups of disease states to be able to determine to any extent the distribution of anti-nuclear factor in disease and in normal individuals. The main disadvantage of this method is the failure to distinguish between the various different types of anti-nuclear factor (see later in discussion) and the variation in sensitivity in different hands.

Whilst the indirect methods employing tanned cells and latex and bentonite particles give less consistent results than the fluorescent antibody technique they have the advantage of being relatively simple titration methods in which comparisons can be made between different positive sera. The titres, as in the case of the fluorescent antibody method, tend to be high in Systemic Lupus Erythematosus, and low in the occasional positives found in the miscellaneous disease groups. These coated cell or particle methods suffer from the disadvantage that the antigens used are insoluble in normal saline and require to be partly

depolymerised in order to achieve solubility (with possible alteration in reactivity). In addition, D.N.A. is an acid substance with a pH of 6.5-7 and the pH must be kept above 7 to avoid non-specific reaction between D.N.A. and serum protein. Latex particles are themselves agglutinated directly by certain types of gamma globulin, for example, the macroglobulins in the sera of patients with Rheumatoid Arthritis. Positive reactions may therefore appear unassociated with anti-nuclear factor. These difficulties may explain the wide variation in incidence found using this technique (see Table 20).

The direct identification of antigen reactions by complement fixation has the advantage that it permits the satisfactory analysis of the reactivities of the various constituents of the cell nucleus, and that it is also a titration method. The anti-complementary activity of many Systemic Lupus sera, however, can cause difficulty with this test.

The failure to obtain a positive test in the majority of treated cases, as already discussed, makes the precipitin test unsuitable for the study of the incidence of anti-nuclear reactivity, but it has been of use in the study of the characteristics of the reaction. (See page 153).

The nature of the anti-nuclear factor

In the present work the nature of the anti-nuclear factor has been studies in 24 sera. The ammonium sulphate fractionation procedure provided very little useful information except to show that the factor, like serum globulin, withstood precipitation by a 50% solution. Elution from electrophoresis paper was useful in that no activity was found in the pure albumin, alpha 1 and alpha 2 fractions which were obtained (in the case of the 2 S.L.E. sera P and H and the liver serum Hu examined). It also showed the activity of S.L.E. serum P restricted to the pure gamma globulin. Whilst S.L.E. serum H also showed activity in this pure gamma fraction, additional activity occurred in the (gamma + beta) fraction both by this method (Table 11) and by chromatography (Page 65) (since the "gamma globulin" fraction had been shown by immunoelectrophoresis to contain some beta globulin (Plate 9)). Mercaptoethanol results excluded the possibility that these two S.L.E. sera contained any appreciable activity in the beta 2 M macroglobulin fraction (19 S), and the ultracentrifuge result was consistent with this finding. All the other S.L.E. sera subjected to mercaptoethanol treatment were unaffected (Table 13), i.e. their activity was 7 S gamma globulin. Thus it can be concluded that in Systemic Lupus (at least in the 8 sera tested) the anti-nuclear factor is an immunoglobulin of the 7 S type. The finding that the anti-nuclear factor is a 7 S gamma globulin agrees with numerous studies on the nature of the L.E. cell factor. Thus using salt precipitation and electrophoresis Haserick (1950) and Haserick et al.(1950).

localised the factor in the gamma globulin. Using zone electrophoresis, Carlson and Mollerberg (1958) came to the same conclusion and by means of this technique and cellulose chromatography Fallet et al. (1958), Willkens et al. (1958), and Scheidegger et al.(1960) confirmed these findings. Holman and his co-workers (1957, 1959) showed L.E. cell activity in the 7 gamma globulin fraction by zone electrophoresis and ultracentrifugation. There have been few reports of fractionation studies using anti-nuclear activity other than L.E. cell formation. Willkens et al. (1958) (op.cit.) used agglutination of D.N.A. coated latex particles in addition to L.E. cell formation in the study quoted above, and found that the factor responsible for this activity also occurred in the "pure gamma globulin" fraction. Seligmann (1959) reported the specific inhibition of precipitin lines against D.N.A. by addition of anti-human gamma globulin to lupus sera. Goodman et al. (1960) studied 6 sera from patients with Systemic Lupus by means of chromatographic and ultracentrifugal procedures. They confirmed their earlier work (1959) showing L.E. cell activity in the gamma globulins, and further showed that anti-nuclear activity against mouse liver nuclei, as demonstrated by the fluorescent antibody method, was present in the non-macro 7 S gamma globulin fraction of 3 of the sera they studied. Three other sera contained anti-nuclear activity against mouse nuclei in fractions corresponding to both the non-macro and the macro globulins. However, no immunoelectrophoretic results were shown to exclude the possibility that the "remaining globulins" (called "primarily macroglobulins" by these workers) might contain gamma globulin of the 7 S type. In the

present work, when chromatography of human serum was carried out, only part of the gamma globulin was eluted with the low ionic strength buffer used (0.0175 M. phosphate, comparable with the 0.02 M phosphate buffer used by the above workers). Electrophoresis of the "remaining fractions" clearly indicates that it contains gamma globulin.

In contrast, the present work has shown that in the case of the rheumatoid arthritis sera, the factor can be present as either 7 S or 19 S globulin or as a mixture of the two (Tables 12 and 13). The three sera unaffected by thiol treatment and therefore judged, like the Systemic Lupus sera, to be primarily 7 S globulin, had no LE. cell forming activity. In the case of rheumatoid arthritis serum Ha. which appeared to contain a mixture of both immunoglobulins, the 7 S fraction was demonstrated by chromatography (page 65) and confirmed by the ultracentrifuge study (Table 12), whilst the 19 S was deduced from the reduction in staining after thiol treatment. The activity in the other disease groups studied shows the same trend as that in Rheumatoid Arthritis (Table 13) although the numbers tested are too small for generalisation at present. The findings in the present work that anti-nuclear activity may be present in the macroglobulin component of sera from some cases of Rheumatoid Arthritis, Liver and Thyroid Disease, is of interest in view of the reported finding of antibody activity in this fraction against a variety of other antigens (Grubb and Swann 1958). These workers used thiol reducing agents to destroy macroglobulin (confirmed earlier observations using ultracentrifugal techniques) and showed that complete anti Rh

antibody was of macroglobulin character in contrast to incomplete anti Rh antibody which was non macroglobulin. They also showed that the human iso-agglutinins anti A and anti B were of macroglobulin type as also was Paul Bunnell antibody. The possibility that macroglobulin represents an immature form of antibody response (Laurell 1961) may or may not apply to the Rheumatoid Arthritis cases. It is interesting to speculate on a possible relationship between type of Rheumatoid immunoglobulin and the likelihood of developing Systemic Lupus. Further consideration of the results in the present work shows (Table 14) that the sera which can be absorbed with nucleoprotein may have either 7 S or the 19 S type antibody. Thus there would appear to be no relation between the antigen and the globulin type with which it reacts. This observation is similar to that of Biswas (personal communication) in human sera giving biological false positive tests for syphilis. This variation in the globulin response to the same antigen in different individuals is consistent with an abnormality of the antibody forming cells considered later in the discussion.

The nuclear components involved in the anti-nuclear factor reaction

Evidence indicating the particular nuclear components involved in the reaction with anti-nuclear factor positive sera is provided by two main lines of study in this work. The absorption studies with calf thymus nucleoprotein, D.N.A. and histone, was carried out on a group of sera representing the four main groups of cases which the clinical study has shown to contain numbers of anti-nuclear factor positive cases in excess of that in the normal control series. The histochemical studies, on the other hand, were carried out mainly with sera from cases of Systemic Lupus. In this study, a variety of enzymes were used and their effect on the cell nuclei was correlated with the ability of the nuclei to take up anti-nuclear factor.

Systemic Lupus sera

The results with the group of sera which were used in the absorption study indicated that the addition of calf thymus nucleoprotein removed some or all of the activity in all cases, and it has been shown that this absorption was specific for anti-nuclear factor. The addition of D.N.A. to the sera was shown to have no effect on the anti-nuclear factor activity and histone removed the activity in a non-specific manner, as would be expected from its strongly basic character. The histochemical studies showed that the removal of D.N.A. by D.N.A'ase rendered the nuclei of tissue sections incapable of taking up antinuclear factor. Removal of histone by citric acid

had the same effect in spite of the fact that D.N.A. was shown to have remained in the nuclei. Incidentally, it was shown that after anti-nuclear factor was attached to nuclei, it remained in situ despite the subsequent removal of D.N.A. or histone or both. The evidence obtained from the work with sperm nuclear material showed that mature sperm, which contain protamine instead of histone attached to the nuclear D.N.A. (Vendrely et al. 1957) failed to take up anti-nuclear factor and this suggests that the anti-nuclear component reacted in this group of sera with a complex of D.N.A. and histone rather than with either substance individually. The results of the absorption and enzyme studies support this possibility.

As seen from the animal experiments, the nucleoprotein preparation used in the absorption studies contained traces of protein constituents in addition to the D.N.A. and histone components. The possibility that such a contaminating material may be the antigen which which absorbed the anti-nuclear factor from the sera was excluded by the failure of proteolytic enzyme treatment to effect the ability of cell nuclei to take up the factor.

Although in most cases the anti-nuclear factor is directed mainly against a complex of D.N.A. and histone, a small part of the activity appears to be directed against some other nuclear component in the cases which showed persistence of some staining after nucleoprotein absorption. This applies also to those sera of the thyroid and liver disease groups which were unaffected or only partly absorbed by addition of nucleoprotein. All these sera when exposed to D.N.A'ase treated

nuclei gave preparations which showed no nuclear fluorescence. The failure to obtain nuclear staining with these sera may be attributable to loss of sufficient of another nuclear component due to the manipulations involved in the D.N.A'ase treatment. The nuclear component concerned in this reaction may be that described by Holman Deicher and Kunkel (1959). This fraction, extractable from nuclei by isotonic phosphate buffers, gives positive complement fixation tests with most sera from Systemic Lupus patients, has been shown by cross absorption experiments to be distinct from the nucleoprotein antigen. In fact the manipulations in the D.N.A'ase treatment include a wash in isotonic phosphate buffer, and this "phosphate-extractable component" is almost certainly responsible for some of the staining.

Other Diseases

Rheumatoid Arthritis: All the sera tested in this group appear to contain antinucleoprotein factor only (Table 17A).

Liver and Thyroid Disease: Whilst the majority of the sera in this group contain antinucleoprotein factor only (Tables 17A,B), some, as discussed above, contain both this and the "phosphate-extractable" factor. These results are consistent with the observations of Holman Deicher and Kunkel (1959) who showed, using complement fixation and precipitin techniques, that sera from Systemic Lupus patients may contain

globulin factors capable of reacting with four different components of cell nuclei, namely whole nucleoprotein, isolated D.N.A., histone and a buffer extract of cell nuclei (mentioned above). Individual sera were shown by cross absorption techniques to contain either all these factors or various combinations of them. Similar results with some or all of these nuclear components are reported by Robbins et al. (1957), Pearson et al. (1957), Asherson (1959), Aisenberg (1959) and Hijmans and Schuit (1959), also by Friou (1958) who found that artificial complexes of D.N.A. and histone dried on glass slides were suitable substrates for the detection of anti-nuclear factor activity. Of special interest is the serum which failed to fix complement with whole nucleoprotein but was active against nucleoprotein treated with a proteolytic enzyme (Holman et al. (op.cit.)) suggesting that the protein part of the nucleoprotein molecule appeared to be masking the combining sites of the D.N.A. This observation provides an attractive explanation for the one example in the present series of a serum which was negative in the anti-nuclear factor test but which, however, gave a positive L.E. cell test. It is conceivable that in this case the incubation of polymorphs in serum for the L.E. cell test was sufficient to allow the proteolytic enzymes released from polymorphs (Raffel 1961) to expose the reactive sites on the D.N.A. molecule, whereas simple layering of serum on tissue sections failed to achieve this. As has already been mentioned (page 135), the fluorescent antibody method for detecting anti-nuclear factor failed to differentiate between these

different components of cell nuclei. However, during this study high power examination of preparations had shown that occasional sera gave a more speckled type of fluorescence than the usual overall nuclear fluorescence observed. More recently some workers have managed to achieve a more clear-cut differentiation of the type of staining. Beck (1961) in a beautifully illustrated report, describes three types of nuclear staining which he designated "speckled", "homogeneous", and "nucleolar" Rapp (1962) confirmed these observations and found "speckled" chromatin staining in 17 lupus sera, the chromatin and nuclear membrane "homogeneous" type in 9 sera, the "nucleolar" staining in three sera; in addition a fourth type of nuclear membrane staining occurring in 18 of the sera. More recently, Beck et al. (1962) reported on further study of the anti-nucleolar antibody in 11 patients and found that 6 had progressive systemic sclerosis, 2 Sjogren's disease, one discoid lupus, one systemic lupus and one case had pernicious anaemia. The antigen concerned has not yet been identified, but it appeared to lack species and organ specificity thus resembling the other types of anti-nuclear factor. Beck and his colleagues considered that this type of staining may be present in many systemic lupus sera but was obscured by the "homogeneous" overall staining. Finally, Lachmann and Kunkel (1961) claimed that the "speckled" staining occurred in sera in which antibody to "phosphate buffer extract" was the predominant antibody present. No correlation, however, has been attempted between the other types

of staining and any other of the components described by Holman (op.cit.). Since most sera contain an anti-nucleoprotein factor (Kunkel et al. 1960 and present study) and since the fluorescent antibody method detects this factor, it is not easy to comment on how many of the other components of Holman are being detected by this method.

Relationship of the anti-nuclear factor to the L.E. cell factor

The L.E. cell factor often appears in the same sera as anti-nuclear factor, and only in one instance in the present work was it present without the anti-nuclear factor. On the other hand, a substantial number of sera contain anti-nuclear factor in the absence of L.E. cell activity. Physico chemical fractionation procedures have shown in the present work that the factors are present in the same serum component and this is supported by other workers (see page 137). The present work suggests that both D.N.A. and histone in combination must be present in the cell nucleus in order that anti-nuclear factor will react with it, and this conclusion has also been reached by Holman and his colleagues (1957; 1959) with regard to the L.E. cell factor. These workers showed that the latter can be absorbed from serum by nucleoprotein preparations and that this absorption occurred only if both the D.N.A. and the histone were present. Hijmans and Schuit (1958) confirmed that calf thymus nucleoprotein completely or almost completely abolished the L.E. cell inducing ability of sera, and in addition found

that D.N.A. or R.N.A. alone had no effect. The work of Klein et al. (1959) showed that nucleoprotein was active in inhibiting the L.E. cell phenomenon and that the inhibitory effect was lost after treatment of the nucleoprotein with D.N.A.'ase and proteolytic enzymes. Whilst the above reports concur that histone is required for L.E. cell activity the report of Lachmann (1961) is an exception. He described "characteristic lupoid cells" formed using papain-treated sperm heads as the substrate, in spite of the fact that these contain protamine and not histone combined with D.N.A. This finding might have been peculiar to the particular serum he used. In contradistinction to his finding, the author was unable to show anti-nuclear staining by a strongly positive serum of a preparation of papain-treated sperm heads provided by Dr. Lachmann.

Most workers agree that after absorption with D.N.A. alone L.E. sera keep a great part of their capacity to induce the L.E. cell phenomenon, but there is a discrepancy in reports on the behaviour of the gamma globulin eluates from precipitates formed between D.N.A. and lupus sera. Thus Seligmann (1958) reported L.E. activity of such extracts whereas Deicher, Holman and Kunkel (1959) failed to do so.

There would appear to be no clear-cut solution to the problem of accurately defining the relationship between anti-nuclear and L.E. cell forming activity. That each is closely connected with both D.N.A. and histone in combination seems likely. Further study of anti-nuclear positive sera which show no L.E. activity might further elucidate the relationship.

Immunisation experiments with nuclear materials

The failure in the present work to provoke specific immune reactions to nuclear material despite the use of adjuvants and the use of repeated injections in the same animals, agrees with the experience of the majority of workers. Thus Seligmann (1960) immunised rabbits with a prolonged course of D.N.A. either untreated or conjugated to normal rabbit serum by heating of formalinization. The sera obtained gave negative results in complement fixation and precipitin tests. It is of interest that the sera which he obtained following immunisation with calf thymus nucleoprotein did not contain anti D.N.A. antibodies either and only reacted with high concentrations of nucleoprotein suggesting the presence of antibodies to contaminating material. Holman and Deicher (1959) obtained similar results following D.N.A. and nucleoprotein injection in that the reactions which they obtained were all against contaminating material. In extensive immunisation of rabbits with calf thymus nucleoprotein and D.N.A. combined with Freund's complete adjuvant, Lachmann (1961) failed to induce the formation of L.E. cells. Similar failures to induce immune response to nuclear materials were encountered by Friou (personal communication) and Hijmans (personal communication). There have been a number of reports of successful attempts to immunise animals with nuclear components. Earlier reports describe anti-nucleic acid antisera obtained following the injection of a complex mixture of bacterial antigens (Sevag 1938, Heidelberger and Scherp 1939, Menzel and Heidelberger 1938, Pennell 1940). Heidelberger (1940) commenting on

these reports was unable to accept that these findings entirely eliminated the possible role of impurities in the antigens used to detect antibody, particularly as such antigenic contaminants would have been present in the mixture of antigens used for immunisation. Critical examination of the more recent reports of successful immunisation with "purified" nuclear materials reveals similar difficulties in the way of accepting the purity of the nuclear preparations and the specificity of the antisera. Blix et al. (1954) reported that they obtained complement fixing and in some instances, precipitating antibody to a variety of D.N.A. preparations and also to calf thymus nucleoprotein. They failed to exclude the possibility that the antibody response occurred as a result of contaminating protein material in the preparations which acted as the antigen, since the chemical tests they used would not necessarily detect the trace amounts of protein required for successful immunisation. The reduction of the antibody response following depolymerisation of the D.N.A. which they report can be explained in one of two different ways. Either the suggested protein antigen was simultaneously broken down with the D.N.A. and thus lost its antigenicity, or the D.N.A. was acting as an adjuvant (Snell 1957) enhancing the antibody response to a minor contaminant of the preparation, and depolymerisation prevented this adjuvant effect.

It is of interest that in the experiment of Phillips et al. (1958) multiple precipitin bands appeared in the gel diffusion tests with the "purified" D.N.A. and the antisera obtained after immunisation with this material. It is suggested that a contaminating antigen may require the

presence of D.N.A. without which it would form only soluble complexes with antibody. They also suggested that D.N.A. may be co-precipitated with the contaminating antigen and would thus appear in the precipitate. Some of the criticism already raised can be levelled also at 2 recent reports which claim successful immunisation with D.N.A. Colter and Ellem (1961) found complement fixing antibodies in the sera of rabbits immunised with D.N.A. from mouse liver and Ehrlich ascitis tumour cells. The report of Miescher et al.(1960) described experiments in which groups of rabbits and guinea-pigs were repeatedly immunised with Brucella abortis nucleoprotein, calf thymus nucleoprotein, and cell nuclei given together with Freund's complete adjuvant. No precipitins were obtained with any of these antigens but complement fixation (using the very sensitive conglutinating complement absorption test) and passive cutaneous anaphylaxis tests gave positive results in some animals with nucleoprotein, D.N.A. and histone. There was interspecies cross reactivity and D.N.A'ase treatment of the D.N.A. abolished its serological reactivity in the tests, except however when 128 gamma or more D.N.A. was used. With these larger quantities of antigen D.N.A'ase treatment failed to alter the reactivity of D.N.A. in the tests suggesting the presence of a contaminating antigen. Treatment of the D.N.A. with the proteolytic enzyme trypsin did not affect the reactivity of the preparation in the complement fixation tests. However these experiments and those reported above failed to include a test of the antisera against possible contaminating materials

obtained during the preparation of the D.N.A. such as the saline soluble protein material used for skin testing in the present work. In addition, other non-protein substances are considered to be present in D.N.A. preparations (see Lachmann 1961) and the possibility exists that these results may be due to such an antigen. Thus the report of Levine et al. (1960) proposed that the "anti-D.N.A." antibody, formed in rabbits immunised with T 4 phage D.N.A., was in fact reacting with glucosylated 5 - hydroxy cytosine. This was deduced from the ability of the antibody to cross react only with a closely related phage with a similar high glucose content. It should be noted, however, that this antibody does not cross react with calf thymus D.N.A., and thus appears to be different from that described by Miescher. Other materials which may contribute to the antigenicity of nuclear materials are the chromosome bound protein "chromosin" or "chromosomin", an alkali-soluble lipoprotein and a globulin (Busch and Davis 1958). In conclusion, the numerous complex protein and other components known to be associated with the cell nucleus and the possible contamination of D.N.A. and nucleoprotein preparations with such material leads to considerable sources of error in any report which claims the successful induction of antibody to nuclear material. Finally, as D.N.A. is part of all nucleated cells it would seem unlikely that a normal animal could be induced to react to and form antibody to such an important cell constituent. The more likely explanation for the formation of the anti-nuclear antibodies found in systemic lupus is that they are produced by abnormal antibody producing cells in the manner discussed in another section.

The nature of anti-nuclear factor in relation to antigen-antibody reactions

The reaction between sera containing anti-nuclear factor and nuclear material has been shown using a number of standard immunological techniques (Table 20) to result in the fixation of complement, the agglutination of particulate materials coated with the specific antigen, and the precipitation of soluble antigen. In addition, the anti-nuclear factor has, in common with other known antibodies, been isolated in the gamma globulin and macroglobulin fractions of the serum, and it can be detected by the anti-globulin consumption test and by the fluorescent technique using an anti-human globulin conjugate. As has been shown, the most efficient conjugate for this purpose was one showing one main precipitin band against the gamma globulin component on immunoelectrophoresis (plate 5), and it is also known that antibody to gamma globulin cross reacts to $beta_2$ macroglobulin (Crowle 1961).

No completely satisfactory criteria exist for defining an antigen antibody reaction and each of the above-mentioned features of the primary and secondary effects of antigen antibody union by itself can be reproduced by non-antibody antigen systems. For example, it has been shown that heat denatured gamma globulin and aggregated gamma globulin are able to fix complement (Ishizaka et al. 1959) (Christian 1959), and sera which have stood at room temperature for a few days are well known to become anti-complementary (Zinsser and Johnson 1911). The agglutination of red cells by certain viruses and phytohaemagglutinins is

a similar type of non-immunological phenomenon as is the finding of Liesegang rings in double diffusion in agar due to inorganic materials (Feinberg 1957), and the precipitation of D.N.A. by proteins (Goldwasser and Putnam 1956) (Coleman and Edelhoch 1956). One of the most commonly used criteria for deciding on the immune nature of a reaction is the shape of the precipitin curve in the quantitative precipitin reaction. In the case of classical antigen antibody systems, less precipitate is formed in both the antigen and the antibody excess zones and this procedure was used to study the anti-nucleoprotein reaction., When increasing quantities of nucleoprotein were added to a constant amount of nucleoprotein-precipitating serum no decrease in the quantity of precipitate was found with the higher concentrations of nucleoprotein (Figure 5). The evidence from dextran-anti-dextran systems that higher molecular weight antigens form complexes with antibody, which are less soluble in antigen excess than those from smaller molecules (Kabat and Mayer 1961) was shown not to apply to the high molecular weight substance D.N.A. by Deicher et al. (1959), and cannot therefore be assumed for nucleoprotein. The most likely explanation lies in the high viscosity of the more concentrated nucleoprotein solutions which would interfere with their adequate mixing with the antibody and thus effectively prevent much of the antigen from making contact with the antibody molecules. The coating of the coils of nucleoprotein noted in the capillary tube precipitin tests supports this contention. It was

to overcome this that nucleoprotein was used in a lower concentration which was kept constant while the concentration of the serum was varied (Fig. 6). When the results are presented to show the precipitate formed with increasing quantities of antigen per unit of serum (Fig. 7), a curve of the classical type is obtained. Similar precipitin curves with D.N.A. and serum from patients with Systemic Lupus were obtained by Deicher et al. (1959) and Barbu et al. (1960). Deicher and his co-workers also established that the reactions could be distinguished from the non-immune precipitin reactions obtained at a pH of 5.1 described by Colman and Edelhoch (1956) and Goldwasser and Putnman (1956) since the immune type of reaction was shown to occur within the pH range 7.5 to 8.6. In the present work, the pH of the reaction mixture was 8.0. In addition these workers were successful in demonstrating a precipitin band with D.N.A. and serum from lupus patients, using the technique of immunodiffusion, similar to the result shown in the present work (plate 11). In both cases the position of the precipitin band was shown to depend on the concentration of the antigen - as would be expected in an antigen-antibody reaction.

It is the co-existence of numerous immunological features described above which supports the thesis that anti-nuclear factor is an antibody. At the beginning of this Chapter, and in the Introduction (pages 16 and 20), mention has been made of antibodies to red cells, white cells, platelets and certain cytoplasmic constituents. It seems justifiable, in view of the evidence discussed above, to include the anti-nuclear factor in this group of antibodies, and with this assumption, the final part of the discussion will deal with its possible mode of origin and its pathogenetic significance.

The role of the anti-nuclear factor in disease

The activity of the red cell antibody in autoimmune haemolytic anaemia is clearly indicated by the episodes of haemolysis, and the uptake of the antibody on the red cells can be shown readily by the direct Coombs test. This contrasts with the difficulty in demonstrating the in vivo activity of anti-nuclear factor in Systemic Lupus, shown in the present work by the fact that sections of kidney and spleen from two patients with high titres of circulating anti-nuclear factor showed no evidence that uptake of the factor by the nuclei of the cells had occurred in vivo (plate 15). These nuclei, however, were able to take up the factor when the sections were layered with autologous serum, i.e. in vitro. Similar results are reported by Holman et al. (1959), Mellors et al. (1957) and Vasques and Dixon (1957). The finding in one case by Alexander and Duthie (1958) that the nuclei of skin sections from a patient with the disease were unable to take up the factor in vitro suggested to them that the nuclei were already coated with antibody, but there have been no other reports of similar findings. Robineaux (1959) found that living polymorphs kept in vitro can take up the L.E. cell factor(in contrast with Lachmann's failure to show this, (Lachmann 1961) from the serum in which they are suspended. Robineaux' finding, however, is open to the objection that the cells were already dying due to the unsuitability of the environment. That the uptake in vivo is unlikely is supported by the difficulty in demonstrating that

L.E. cells have formed in the peripheral blood, as indicated by the rare reports of such a finding (Watson et al. 1951, Sickley et al.1955, and Chomet et al. 1953). That the factor is unlikely to attack living cells is shown, too, by the failure of the factor to affect the growth of cells in tissue culture (Rapp 1962, Lachmann 1961) (Hijmans personal communication),(Baum personal communication). Some preliminary experiments were carried out by the author in co-operation with Dr. G. Loewi using tissue cultures of guinea-pig testis and indicated that the serum from Systemic Lupus patients had no effect on either the morphology or growth of the cells. It was of particular note that the anti-nuclear factor was taken up on air dried films of these cells only after they had been damaged by exposure to dilute trypsin. This is probably similar to the exposure of the nuclei in cells cut across in tissue sections (i.e. damaged) and the fact that not all cells are cut through in such sections might explain why not every nucleus can be shown to be stained (plate 15). The experiment of nature whereby the infants of mothers with Systemic Lupus receive L.E . cell factor through the placenta and yet show no signs of the disease, indicates that the presence of the factor for a few weeks in the circulation of the infant is not able to produce clinical signs of the disease(Berlyne et al. 1957, Bridge and Foley 1954, Burman and Oliver(1958)

The important observation in Systemic Lupus which makes it necessary to consider further the question of the in vivo uptake of anti-nuclear antibodies is the well recognised presence of altered nuclear

material in the lesions of the disease. This is shown by the haematoxylin staining bodies found in the tissues of patients with the disease (Gross 1932, Ginzler and Fox 1940 (Klemperer op.cit.). These are described as reddish purple amorphous structures usually ovoid or spindle shaped, about the size of a cell nucleus, occurring singly or in clusters or coalesced into aggregates (Worthington et al. 1959). They are considered to be derived from cell nuclei and have been shown to contain nuclear material in combination with larger quantities of protein than is normally combined with the D.N.A. of cell nuclei (Godman and Deitch 1957, Godman et al. 1958). These workers believed that the antibodies to nuclear materials displace the nuclear histone from combination with the D.N.A. to form both the L.E. inclusion body and the haematoxylin bodies of Systemic Lupus. In view of the results of the present work it seems most likely that this displacement would occur in the nuclear material of dead cells. If the site of cell destruction was in the lumen of blood vessels, this could lead to the lodging of aggregated complexes of antibody with nuclear material in small blood vessels. This would be comparable to the finding of German (1958), that haematoxylin bodies can be demonstrated in the renal glomeruli of rabbits following the injection of human L.E. cells. This is supported by the observations of Dodd et al. (1959) that Feulgen positive material in the serum of patients with Systemic Lupus had a similar electrophoretic mobility to the gamma globulin fraction. This is reminiscent of the association of other globulins with cell components exemplified by haptoglobin bound with haemoglobin. It should be noted at this point that Grabar (1959) speculates that such globulins, including all antibody globulins, are part of a general physiological

mechanism which transports products of metabolism or cell degradation. According to this hypothesis, the appearance of substances referred to as auto-antibodies only represents an exaggerated form of a normal phenomenon elicited by tissue destruction. Grabar cites the example of the naturally occurring cold antibodies which are known to increase after certain infections such as atypical pneumonia (see Dacie 1962). (In this connection anti-nuclear antibodies active only in the cold are reported by Couchman et al. 1961). Although the evidence for this hypothesis remains highly speculative it is valuable in that it draws attention to an alternative explanation for the phenomena under discussion. The observations of the lesions of experimental serum sickness showing fibrinoid necrosis and necrotising arteritis in the coronary vessels of rabbits sensitised to horse serum led Rich and Gregory (1947) to make a comparison between these lesions and those of Systemic Lupus. Later reports have established that the immune phase of antigen elimination is conditioned by the presence of large amounts of soluble antigen-antibody complexes in the circulation, and these observations emphasise that tissue damage may result from the mere localisation in the vascular endothelium of preformed antigen-antibody complexes (Germuth 1953, McCluskey and Benacerraf 1959).

Strengthening the possibility that such complexes exist and may have pathogenic importance in Systemic Lupus is the finding of low serum complement levels in these patients (Ellis and Felix Davies 1959, Asherson 1959) and the association between complement fixation and the

increased capillary permeability of the immediate hypersensitivity type (Ishizaka et al.1959) (that is if the low levels of complement have not resulted from impaired protein synthesis or loss through damaged kidneys). Finally, it is necessary to consider the fact that "systemic lupus like disease" and classical Rheumatoid Arthritis can exist in patients with marked hypogammaglobulinaemia (Good and Rotstein 1960) in whom the ability to form circulating antibodies is limited but who do, however, have an intact mechanism allowing the development of the tuberculin type "cellular immunity". This latter fact is of considerable interest in view of the high incidence of skin reactions to ingested leucocytes in systemic lupus patients (Bennett and Holley 1961, Friedman et al. 1960). These reactions have some of the features of the tuberculin type response, in that they appear at 24 hours and show histologically a monocytic cellular infiltration, but also include the polymorph infiltration typical of the immediate type of response. The unusally high incidence of the "rheumatoid diseases" in the hypogammaglobulinaemic patients is of some interest in view of the possible protective role of circulating antibody. The work of Kaliss and his colleagues on homograft rejection has shown that the recipient (C57 black mice) injected with antigens prepared from mixed normal and tumour tissues from the donor (strain A mice) became susceptible to a transplantable strain A tumour to which they were normally completely resistant. It is considered by these workers that this phenomenon is

dependent on the presence of antibodies in the serum (Woodruff 1960). A possible example in auto-immune disease of such a phenomenon is the prevention of the development of demyelinating encephalomyelitis in guinea-pigs immunised with nerve tissue by the injection of the serum from an affected guinea-pig (Thomas personal communication).

In conclusion, it appears that whilst there is no direct evidence that antibodies against nuclear materials play a part in the induction of the tissue damage of systemic lupus, it seems likely that the effect of antibody on the nuclei of dead cells and the lodging of aggregated complexes of circulating nuclear materials and anti-nuclear factor in small blood vessels might result in activation of substances implicated in the tissue damage associated with sensitivity of the immediate or serum sickness type.

Mode of origin of anti-nuclear factor

Three general mechanisms of auto-antibody production have been proposed. The first and attractively simple assumes that cell components are altered and rendered antigenic by the absorption to their surfaces of some foreign material such as a drug or a bacterial or viral agent or a product of such an agent. The classic example of such a phenomenon is that of sedormid purpura (Ackroyd 1954) in which antibody is produced which can agglutinate platelets in vitro in the presence of the drug, and, with the aid of complement, lyse them. A number of other drugs have been associated in a similar way with purpura, including quinine, digitoxin and sulphamethazine (Waksman 1958). Numerous examples of drug induced haemolytic anaemia and leucopenia have been reported including those due to Fuadin (Harris 1956) and aminopyrine (Krake 1931). The significant fact which emerges from studies on these reactions is that in vitro the antibody can be demonstrated only in the presence of the drug. Thus the antibody has been stimulated not by a change in red cell structure but by a hapten complex of foreign material and normal cell. There is some evidence that auto-immune haemolytic anaemia may be associated with certain bacterial or viral infections, including Newcastle disease and Coxsakie viruses, and that measles virus is associated with platelet disease (Moolten et al. 1953, Betke et al. 1953, Workman et al. 1954, Atta and Sievert 1953). This evidence, however, has been disputed and efforts to isolate the viruses have been unsuccessful (Morgan 1955). Experimental efforts to modify the antigenicity of red cells have been made by immunising animals with

homologous or autologous erythrocytes treated with various enzymes. Thus Dodd et al. (1953) reported observations obtained by immunising rabbits with normal or trypsin treated erythrocytes. The antisera obtained reacted, after absorption with normal red cells, with trypsin-treated erythrocytes and with the red cells of a substantial proportion patients with acquired haemolytic anaemia . Dacie (1962) points out that this may have been due to existing damage to the red cells by the absorption of red cell auto-antibodies, but nevertheless he states that it may be premature to disregard this hypothesis of antigen-alteration altogether as a cause of auto-immune haemolytic anaemia. The proposed alteration in human gamma globulin which is responsible for the stimulation of Rheumatoid Arthritis factor is considered by Glynn and Holborow (1960) to be likely to occur in normal individuals as well. These workers propose that it is not the degree of alteration of the antigen alone which is responsible for antibody stimulation, but an concomitant alteration in the response of the rheumatoid individual. There is no evidence that haptenic interaction occurs in systemic lupus since, although drug sensitivity is an associated phenomenon of systemic lupus, and the sensitivity disappears on withdrawal of the drug as in sedormid purpura, the progress of the disease is unaltered by removing the drug. In addition, the ability of the antibodies in systemic lupus to cross react with antigens from other individuals is inconsistent with the finding that in general in drug induced thrombocytopenia and

haemolytic anaemia, the hapten must be linked to the cell before combination with antibody takes place (Dacie 1962). A second type of auto-immune mechanism postulates the release of so-called "hidden antigens" normally inaccessible to the antibody forming cells, which react to this antigen as if it were foreign material. The classic examples of such antigens are those of sperm antibodies(against which are associated with sterility in males (Rümke 1959), thyroid (discussed in the introduction) and lens protein (an immunological reaction against which is postulated to account for the occurrence of Sympathetic Ophthalmia (Collins 1953). There is no evidence that the antigens involved in the anti-nuclear reactions or those in the other immunological reactions of Systemic Lupus are sequestered in any way from the antibody forming cells. The rare and controversial experimental production of antibodies to D.N.A. and nucleoprotein (already discussed) contrasts with the relative ease of their production to a hidden antigen such as thyroglobulin. In addition there was no evidence in these experimental animals of any lesion resembling those in Systemic Lupus. This contrasts with the well marked lymphocytic infiltration of the thyroid gland in experimental auto-immune thyroiditis(op.cit.) and also with the lesions produced in the brain and spinal cord following the injection of nerve tissue extracts with complete adjuvant (see Introduction), and with similar findings described in auto-immune adrenalitis (Colover and Glynn 1958). Of interest is the occurrence of anti-nuclear factor in a proportion of

cases of lymphadenoid goitre (Weir et al. 1961, White et al. 1961 and Hijmans et al. 1961). White makes the interesting suggestion that "such findings may indicate that there is a small sub-group of cases of lymphadenoid goitre which have arisen in patients (with Systemic Lupus) who have special propensity to form antibodies generally. Thus possessors of this trait may respond with antibody production and with immune cellular infiltration of the thyroid to amounts of escaping antigens which are tolerated by normal subjects". The possibility remains that Systemic Lupus falls into the category of states due to abnormality to the antibody forming cells or as it is sometimes termed "disturbed tolerance disease". A parallel has been drawn between auto-immune disease and the immunologically induced phenomenon of the graft versus host reaction or runt disease in the experimental animal. This results from the intraperitoneal or intravenous injection of homologous spleen cells in new-born mice (Billingham and Brent) (1957) or rats (Woodruff and Sparrow, 1957, Woodruff, 1957). The recipient animal must be genetically incapable of rejecting the donor cells, or be made immunologically neutral by exposure to X irradiation or be immunologically immature. Oliner et al. (1961) studied F_1 hybrid mice genetically incapable of rejecting parental spleen cells as follows:- A parental donor was sensitised to the recipient spleen cells. When parent spleen cells thus sensitised were injected intraperitonically into the F_1 hybrid recipients, these developed weight loss, hair changes, dermatitis, hypothermia and splenomegaly. The laboratory findings included haemolytic anaemia with a positive anti-globulin test,

leucopenia, thrombocytopenia and abnormalities of the plasma electrophoretic pattern including lowered albumin and increased beta globulin. Raised gamma globulin levels although present in some animals was not a consistent finding. The eluates from red cells giving a positive anti-globulin test were found to have the electrophoretic mobility of gamma globulin. No search was made for anti-nuclear factor in these animals, but L.E. cells were not found despite intensive testing. The clinical disease produced in these animals as the result of the action of an acceptable, immunologically competent graft against the host bears many similarities to Systemic Lupus. Thus the multiple disorders found in the latter condition and noted in Chapter 2, include haemolytic anaemia, positive Coombs test, leupopenia, thrombocytopenia and splenomegaly. The suggestion made by Oliner et al. (op.cit.) that auto-immunisation need not imply a change in normal body components, but rather a change in the antibody forming apparatus of the individual provides the most likely explanation of the underlying abnormality in Systemic Lupus. Under these circumstances, normal unchanged tissues, for example red blood cells, would become antigenic to the aberrant lymphoid system.

There are similarities, too, between runt disease and the lymphoproliferative disorders, chronic lymphatic leukaemia and lymphosarcoma. Haemolytic anaemia, white cell antibodies and thrombocytopenic purpura have been described in these conditions. (Rosenthal et al. 1955, Wittels et al. 1961). The proliferation of

abnormal antibody forming cells which are capable of forming antibody directed against autologous antigens has been explained in the Burnet clonal theory of immunity as being due to the breakdown of the homeostatic mechanism which normally eliminates those cells which have mutated to being capable of reacting with "self". Where the antibody forming cells themselves become the seat of tumour formation as in the lymphoproliferative disorders, the possibility arises that in the associated high mutation rate of rapidly dividing tumour cells, the antibody forming cells lose an organ specific antigen by "antigenic deletion". That such "antigenic deletion" can occur has been shown in hamster kidney tumour cells and rat liver tumour cells (Weiler 1956) and in human skin cancer and gastro-intestinal tumours by Nairn et al. (1960,1961). In the author's view, it is reasonable to suppose that if such rapidly dividing lymphoid tumour cells become isolated temporarily from normal cells containing the deleted antigen, by proliferating for example within a barrier of fibrous tissue, a random mutation capable of reacting to the deleted antigen would not be eliminated. In this hypothetical situation the tumour mutants are considered to be protected in a way comparable to the surviving corneal transplant isolated from the effects of the host lymphoid cells by the avascular cornea (Woodruff 1960). Breakdown of the barrier at the stage when the mutant cells were no longer vulnerable to antigenic elimination, would result in stimulation of the mutants to produce antibody against the normal body cells possessing the deleted antigen,

including normal lymphoid cells. Kaplan and Smithers (1959) evoke this mechanism to explain systemic phenomena in some Hodgkins patients (before there was much widespread involvement of tissues) such as the multiple infections, poor immune response and the acute terminal illness, often fatal, at an apparently early stage of the disease. The fact that lymphoproliferative disease does not necessarily follow such a course can be explained by the failure of the appropriate mutant cell capable of reacting against self to emerge.

Whether "antigenic deletion" plays any part in the sequence of immunological events in Systemic Lupus is an open question. It may be relevant in view of the implication of histone in the anti-nuclear reactions, to note that the histone of tumours is thought to differ from those of normal tissue (Cruft personal communication). On the other hand, evidence is accumulating which suggests that genetic factors are of major importance. There is a significant familial incidence of the disease (see Harvey et al. 1954, Leonhardt 1957), and elevation of the gamma globulin is found in siblings (Pollak et al. 1960) in addition anti-nuclear factor occurs in a higher proportion of relatives of patients with Systemic Lupus than in normal control groups, described by the author together with Ansell and Johnson (1961) by Morteo et al. (1961) and by Mandema et al. (1961).

A very valuable experimental model for the study of auto-immune disease which appears to depend on an inherited weakness of the homeostatic mechanism, is an inbred strain of mice NZB/Bl which spontaneously develops signs of an auto-immune type of

haemolytic anaemia between 5 and 8 months of age (Holmes, Gorrie and Burnet 1961) (Burnet 1962). The direct Coombs test is positive in affected animals, and the auto-antibody state can be transferred to young NZB/Bl mice by the transfer of 20 x 10^6 nucleated spleen cells from an affected mouse. Pathological examination showed splenic enlargement and gross chronic nephritis with hyaline change in the glomeruli and lymphocyte and plasma-cell infiltration of kidney tissue. Most significantly, the thymus glands of the mice showed medullary hyperplasia with considerable lymphocytic proliferation and numerous plasma cells, reminiscent of the changes which occur in Myasthenia Gravis. The role of the thymus in homograph rejection has been emphasised by Miller (1961) and Miller, Marshall and White (1962) The recent striking report of White and Marshall (1962) shows the very interesting connection, already suspected on clinical grounds (Harvey et al. 1954) between abnormality of the thymus and Systemic Lupus by demonstrating anti-nuclear antibodies in 6 of 13 patients with Myasthemia Gravis. These workers point out the association of thymic abnormalities with possible auto-immune diseases such as acute haemolytic anaemia, leucopenia, thrombocytopenic purpura, and suggest that their evidence further supports this association. They demonstrate the possibility that the thymus is the seat of production of antibody in these patients by means of the fluorescent antibody technique, showing the localisation of gamma globulin within the cells of the germinal centres.

The above finding provides experimental evidence for the speculation of Burnet (1962) who, assuming that thymic lesions of the type described above are present also in auto-immune disease other than Myasthemia Gravis, offers a working hypothesis regarding the

role of the thymus in immunity. He regards the thymus as the site of the primary homeostatic mechanism and believes that the essential defect (in the NZB/Bl mice) and in human auto-immune disease is a genetically based lability as a result of which occasional lymphocytic stem cells develop a resistance to the normal intrathymic control. Such a cell would then start producing anti-body while still in the thymus, and should a mutant have arisen which can react to a body component present in the thymus, the gland will take on the appearance associated with immunological reactivity, that is, germinal centres and accumulation of plasma cells. Mature daughter cells, passing into the general circulation, will be resistant to the high concentration of cell antigen and will proceed to manufacture auto-antibody. Burnet's assumption that there may be thymic lesions in a variety of auto-immune conditions remains to be proved, but the findings of White and his colleagues are most significant in this respect indicating an association between a thymic disorder and Systemic Lupus Erythematosus. This hypothesis in Burnet's view "has at least the advantage of calling forth a wide variety of experimental approaches as well as holding out some promise for practical medicine".

It may be true that "we no longer find ourselves lost on a boundless sea but that we have already caught a distinct glimpse of the land which we hope, nay, which we expect, will yield rich treasures for biology and therapeutics", to echo Ehrlich's apt allusion to Bacon in the Croonian Lecture of 1900.

SUMMARY

The study of a factor which can be demonstrated using Coons indirect fluorescent antibody technique, in the serum of patients with Systemic Lupus Erythematosus (and some other diseases) has been described.

An outline of the historical development of the concept of auto-immune disease has been presented, incorporating the technical advances which assisted in elucidation of auto-immune phenomena and an indication of the possible relevance of these to systemic lupus. The main technique used in the study, fluorescence microscopy, was described in detail. A study of the sera of 845 individuals revealed the presence of the factor in 62 of 63 cases of systemic lupus (58 of these were L.E. cell positive), 19 of 132 cases of Rheumatoid Arthritis, 14 of 110 cases of Thyroid Disease, 5 of 39 cases of Liver Disease, 10 of 75 cases of Discoid Lupus, and 5 of 258 cases in the control groups.

Quantitation of the factor by serial dilution showed that the majority of systemic lupus sera had a high titre, while the remaining diseases showed moderate or low amounts of activity.

A comparison with other reported series revealed a variation in sensitivity of the test in different hands, and the possible interpretations of occasional low titre findings were outlined. The

results with the indirect fluorescent antibody technique were compared with other ways of showing anti-nuclear activity. Histochemical, absorption and precipitation studies on sera with anti-nuclear activity suggest that the nuclear antigen concerned in most instances was a complex of D.N.A. and histone, and a similarity to the L.E. cell factor was noted in this respect. It was concluded that the fluorescent antibody technique whilst not able to distinguish easily between the reactions to the several nuclear components which have been implicated, was a sensitive and reliable method of detecting the factor in human sera, and that it would be difficult to sustain a firm diagnosis of systemic lupus in the absence of a positive test.

A study of the nature of the factor by electrophoretic, chromatographic, ultracentrifugal and thiol degradation methods, indicated that it was an immunoglobulin of the 7 S type in the sera from the cases of systemic lupus examined and that it was either of this type or of 19 S macroglobulin type in Rheumatoid Arthritis, Liver or Thyroid Disease; a few sera from Rheumatoid Arthritis cases appeared to contain a mixture of the two types. These findings were compared with reports of similar studies, stressing the importance of analysing the "pure" fractions obtained by these procedures with sensitive immunodiffusion techniques.

Injection of guinea-pigs and rabbits with nuclear materials was, in common with the experience of a number of other workers, without effect in stimulating immune response specific for D.N.A. or histone. The reactions to contaminating materials were discussed and the findings reported by other workers considered in relation to these contaminants. The contention that the anti-nuclear factor is an antibody was evaluated by correlating the results of the different procedures used in the present work and relevant features reported by other workers, and it was concluded that there is strong evidence to support this notion. An analysis of the clinical and serological features of anti-nuclear positive cases in systemic lupus was consistent with the hypothesis that immune phenomena are occurring in this disease. The demonstration of the antibody nature of anti-nuclear factor particularly strengthens this likelihood, since anti-nuclear factor is so constant a finding in the disease. The significance of a positive anti-nuclear factor test in other diseases is discussed, and it is proposed that there is a special relationship between such patients and systemic lupus, related to some abnormality of the antibody forming organs. An assessment of the role of the anti-nuclear reactions in disease was made, not giving the circulating antibody a primary role but stressing the part of the factor complexed with nuclear material of

dead cells and the immediate hypersensitivity type of reactions.

The possible mode of origin of the underlying abnormality of which anti-nuclear factor is the expression was discussed. Setting aside the concept of antigen alteration, it is proposed that there exists in Systemic Lupus Erythematosus an alteration in the antibody forming cells which is genetically conditioned and may be associated with deranged thymic control of the homeostatic mechanism.

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APPENDIX 1.

Criteria for the selection of cases of systemic lupus erythematosus

Obligatory : The sedementation rate must be above 20mm. in 1 hour, Westegren or Wintrobe.

Major criteria: 1) Rash compatible with disseminated lupus.
2) Leucopenia below 5,000 per cu.mm.
3) Finding of LE cells in 15 minute search of 1 slide.

Minor criteria: 1) Arthralgia or effusions into joints.
2) Serositis.
3) Fever above 99° F (37.2°C.) twice in 24 hours.
4) Retinal changes.
5) D.A.T. 1/16 or more.
6) Albuminuria above 5mg. per 100 ml.

For the admission diagnosis the patient must have the obligatory criterion plus two or more major criteria, or the obligatory criterion plus one major and at least two minor criteria.

APPENDIX 2.

Extract from 1958 Revision of Diagnostic Criteria for Rheumatoid Arthritis.

Bull. Rheum. Dis. IX.: 175

Classical Rheumatoid Arthritis

This diagnosis requires seven of the following criteria. In Criteria Nos. 1 through 5 the joint signs or symptoms must be continuous for at least six weeks.

1. Morning stiffness.

2. Pain on motion or tenderness in at least one joint (observed by a physician).

3. Swelling (soft tissue thickening or fluid, not bony overgrowth alone) in at least one joint (observed by a physician).

4. Swelling (observed by a physician) of at least one other joint (any interval free of joint symptoms between the two joint involvements may not be more than 3 months).

5. Symmetrical joint swelling (observed by a physician) with simultaneous involvement of the same joint on both sides of the body (bilateral involvement of midphalangeal, metacarpophalangeal or metatarsophalangeal joints is acceptable without absolute symmetry). Terminal phalangeal joint involvement will not satisfy the criterion.

6. Subcutaneous nodules (observed by a physician) over bony prominences, on extensor surfaces or in juxta-articular regions.

7. X-ray changes typical of rheumatoid arthritis (which must include at least bony decalcification localized to or greatest around the involved joints and not just degenerative changes). Degenerative changes do not exclude patients from any group classified as rheumatoid arthritis.

8. Positive agglutination test - demonstration of the "rheumatoid factor" by any method which, in two laboratories, has been positive in not over 5% of normal controls - or positive streptococcal agglutination test.

9/-

9. Poor mucin precipitate from synovial fluid (with shreds and cloudy solution).

10. Characteristic histologic changes in synovial membrane with three or more of the following:
marked villous hypertrophy; proliferation of superficial synovial cells often with palisading; marked infiltration of chronic inflammatory cells (lymphocytes or plasma cells predominating) with tendency to form "lymphoid nodules"; deposition of compact fibrin, either on surface or interstitially; foci of cell necrosis.

11. Characteristic histologic changes in nodules showing granulomatous foci with central zones of cell necrosis, surrounded by proliferated fixed cells, and peripheral fibrosis and chronic inflammatory cell infiltration, predominantly perivascular.

Definite Rheumatoid Arthritis

This diagnosis requires five of the above criteria. In Criteria Nos. 1 through 5 the joint signs or symptoms must be continuous for at least six weeks.