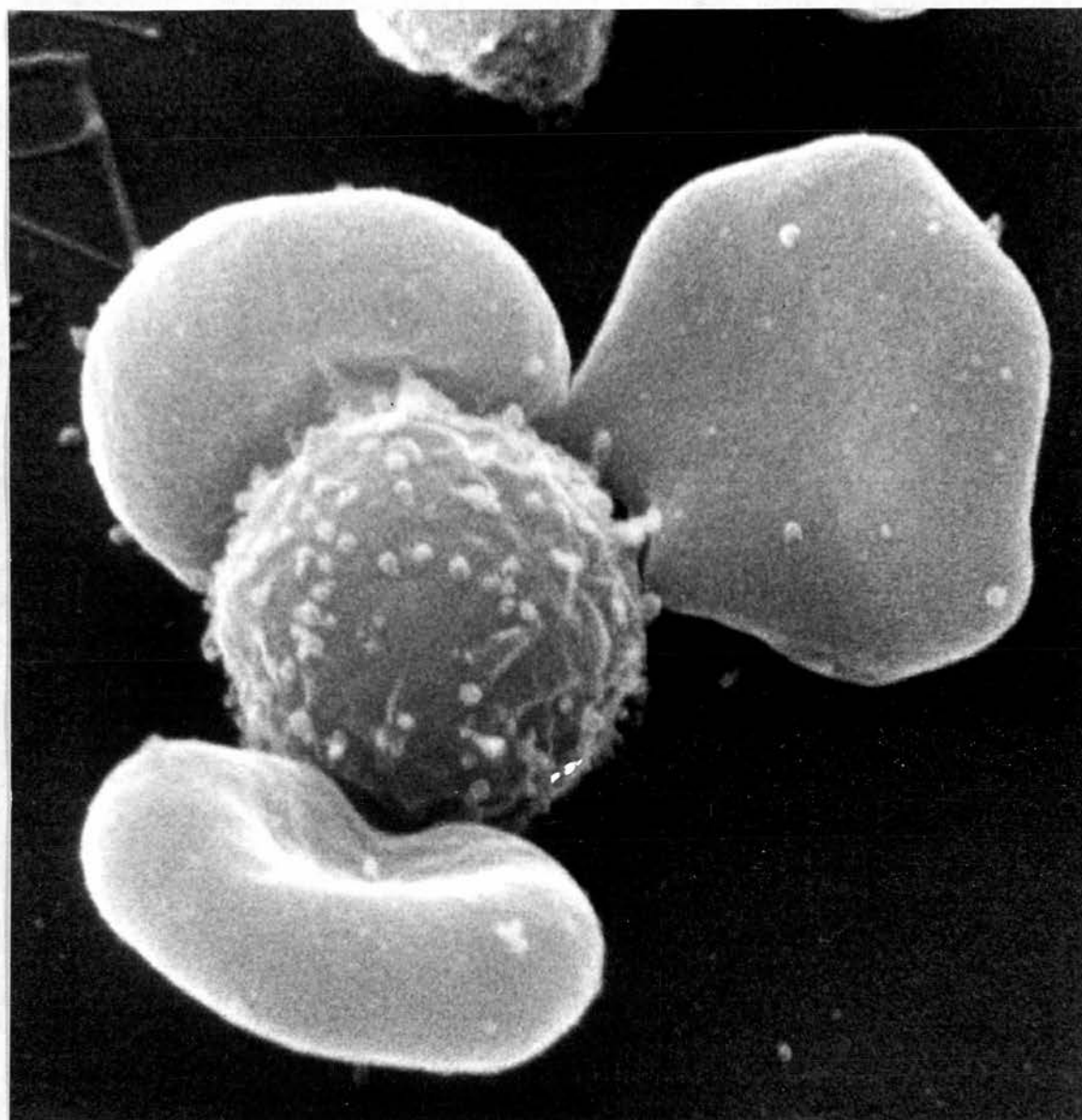


CLINICAL AND EXPERIMENTAL STUDIES ON THE
INTERACTION OF LEUCOCYTES AND ERYTHROCYTES AND
THE EFFECT OF CORTICOSTEROIDS ON SUCH INTERACTION,
WITH SPECIAL REFERENCE TO HUMAN AUTO-IMMUNE
HAEMOLYTIC DISEASE.

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FRONTISPIECE A scanning electronmicrograph
of a homologous rosette from auto-immune
haemolytic anaemia. (x 25,000).

Acknowledgements

Without the co-operation and help of many individuals the work reported in this thesis would not have been possible.

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Dr./

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The main people to have suffered, especially over the last eight to nine months, have been my family, mainly my wife and children. I hope this period has now, at least temporarily, come to an end.

ABSTRACT

In this thesis it has been shown that up to 5% of human mononuclear cells, which have been separated on a ficoll-hypaque density gradient, form rosettes with homologous or autologous erythrocytes. The rosettes characteristically were composed of a central leucocyte, to which were attached at least three erythrocytes. In clinically active auto-immune haemolytic disease, homologous-rosetting cells were found to be increased, when compared with control subjects. In nine, of fourteen patients with auto-immune haemolytic anaemia who were investigated over a prolonged period, a correlation was demonstrated between the rosette level in the peripheral blood and fluctuations in the disease process, either spontaneous or therapeutically induced.

Investigation was directed to the nature of the central rosetting cell. In glandular fever and in cultures of lymphocytes stimulated with phytohaemagglutinin, elevated levels of homologous rosetting cells were demonstrated. Evidence from the literature is discussed, and it indicates that such results are compatible with the fact that the rosetting cell is a T-lymphocyte.

This conclusion is supported by further experimental evidence given in this thesis.

Namely/

Namely the rosetting cells did not carry detectable surface immunoglobulin, but did have receptors for sheep erythrocytes, the latter property allowing them to form mixed sheep and homologous erythrocyte rosettes. In addition, investigation of the physical factors affecting rosette formation supports the conclusion that the majority of rosetting cells are T-lymphocytes.

The second section concerns a detailed ultra-structural examination of the rosetting cells from patients with auto-immune haemolytic anaemia and glandular fever, and from phytohaemagglutinin stimulated cultures. The techniques used, in addition to transmission electron microscopy, were immuno electron microscopy and scanning electron microscopy.

From ten patients with auto-immune haemolytic anaemia, 172 rosette forming cells were examined by transmission electron microscopy. Of this total, 144 were lymphocytes, 21 monocytes, 6 polymorphonuclear leucocytes and one a plasma cell.

Of the lymphocytic rosette forming cells, 44% had features thought to be characteristic of activated T-lymphocytes, namely an area of clearing of cytoplasmic organelles from under the plasma membrane and the presence of cytoplasmic microfilaments.

The/

The form of contact between lymphocytic rosette forming cells and erythrocyte was predominantly of a pointlike nature, and in this form of rosette there was little deformation of the attached erythrocyte. The prime distinguishing feature of the monocytic rosetting cell, was the deformation of attached red cells which they caused.

On the evidence given in this thesis the homologous rosetting cell is a T-lymphocyte, and the role such a cell might play in the underlying cellular disturbance in auto-immune haemolytic anaemia is discussed. Discussion is in terms of T-lymphocyte subpopulations and in particular of T-suppressor cells.

The final section of the thesis is concerned with the effect of cortisone acetate on lymphocyte subpopulations and the interaction of IgG coated erythrocytes with monocytes, in vivo and in vitro, in the mouse.

The results presented, show that cortisone acetate reduced the number of lymphocytes in thymus, spleen and lymph nodes. In spleen and lymph nodes, B-lymphocytes were demonstrated to be more sensitive to such therapy than T-lymphocytes. In addition, cortisone acetate in high dosage in vitro had an/

an inhibitory effect on phagocytosis by monocytes. The evidence, from in vivo studies of blood clearance and organ sequestration of ⁵¹Cr labelled erythrocytes, was felt to indicate that cortisone acetate had a qualitative rather than a lytic (quantitative) effect on monocytes. Suggestions are made for further experiments to extend this work.

CONTENTS

CHAPTER I

Introduction pp. 1 - 8.

1. Historical.
2. Recent developments.
3. Prelude to work in this thesis.

CHAPTER II

Materials and methods pp. 9 - 35.

- A. Human pp. 9 - 27.
- B. Mice pp. 28 - 35.

CHAPTER III

Clinical and experimental studies of homologous
and autologous rosetting cells. pp. 36 - 127.

1. Introductory section and review
pp. 36 - 49.
2. Results pp. 50 - 107.
3. Discussion pp. 108 - 127.

CHAPTER IV

Ultrastructural studies of the rosette forming
cell in auto-immune haemolytic anaemia,
glandular fever and phytohaemagglutinin
stimulated cultures. pp. 128 - 208.

- 1./

1. Introductory section and review.
pp. 128 - 149.
2. Results pp. 150 - 192.
3. Discussion pp. 193 - 208.

CHAPTER V

Discussion of the role of the homologous and autologous rosetting cell in the pathogenesis of auto-immune haemolytic anaemia. pp. 209 - 222.

CHAPTER VI

The effect of cortisone acetate on murine lymphocyte subpopulations, monocytes and the blood clearance of antibody sensitised erythrocytes.
pp. 223 - 262.

1. Introductory section and review.
pp. 223 - 230.
2. Results pp. 231 - 249.
3. Discussion pp. 250 - 262.

BIBLIOGRAPHY pp. 263 - 290

Each chapter and each section contains a detailed guide to its contents, immediately preceding it.

CHAPTER I

I N T R O D U C T I O N

Introduction

1. Historical.
2. Recent Developments:
 - i) Auto-antibodies and complement.
 - ii) Antigenic specificity of IgG incomplete antibodies.
 - iii) Extravascular destruction of the erythrocyte.
3. Prelude to work in this thesis.

Introduction

1. Historical

In large part, the investigations described in this thesis are concerned with the human illness which is now called auto-immune haemolytic anaemia (A.I.H.A.). The first description of this disease is thought to have been given by Hayem (1898), under the title, "Ictère infectieux chronique splénomégalique". Although the presence of abnormal haemolysins in the sera was noted as early as 1908 (Chauffard and Troisier), little progress in understanding of this disorder was made until 1945. It was in this year that Coombs et al. showed that erythrocytes, sensitised by incomplete forms of Rh iso-antibodies, were agglutinated by anti-human globulin sera prepared in rabbits against human serum proteins (direct antiglobulin test). This discovery provided a tool for investigation, and in 1946, Boorman et al. and Loutit and Mollison showed that the direct antiglobulin test, in patients with acquired haemolytic anaemia, was positive. This allowed their distinction from congenital haemolytic anaemias in which this test was negative.

2./

2. Recent Developments

i) Auto-antibodies and complement

Auto-antibodies, detected by the antiglobulin test, are the prime reason for the accelerated red cell destruction encountered in A.I.H.A.. In the last two decades, much has been learned of their serological and immunological characteristics.

The structure and the types of immunoglobulin molecule involved, together with their interaction with the complement system, are now reasonably well understood (Engelfriet et al., 1974).

As the antibodies, together with their specificity and complement sensitisation, are alluded to in this thesis for discussion purposes, a brief review of this aspect is now presented.

The majority of the auto-antibodies in A.I.H.A. have been shown to be IgG. Though IgM auto-antibodies and isolated examples of IgA auto-antibodies have been reported, IgD and IgE as antibodies in this disorder have not been found. (Dacie and Worlledge, 1969; Engelfriet et al., 1968). In addition to antibodies, complement components are often detected, either alone or together with antibody, on the erythrocyte (Engelfriet et al., 1974). The method of fixation of/

of complement by IgG antibodies is not known for certain, but one proposal suggests that the presence of two IgG antibodies on neighbouring antigenic sites is sufficient for fixation to occur (Borsos and Rapp, 1965; Humphrey, 1967; Rosse and Parker, 1968).

The attachment of antibody per se does not appear to produce any damage to the erythrocyte. Thus red cells, which have been strongly agglutinated in vitro, may have a substantially normal survival when transfused (Loutit and Mollison, 1946). On the other hand, if the attachment of antibody to the cell is followed by activation of complement, membrane lesions may be produced, leading to intravascular dissolution of the cell, or early components of complement may become bound to the cell membrane, leading to accelerated clearance of red cells from the circulation.

In most cases of A.I.H.A. where complement is activated, there is interruption of the sequence. Complement lysis does not take place, but complement can be detected on the red cell, most often in the form of C3D (Ruddy and Austen, 1971). The latter is an inactive antigenic determinant of C3, which accounts for its reaction with anti-C3 reagents (Engelfriet et al., 1974).

Other/

Other mechanisms than those due to the inactivators described by Ruddy and Austen (1971), must be operative in preventing completion of the complement sequence in some cases, as C5 and C6 have been reported on red cells in A.I.H.A. (Kerr et al., 1971).

ii) Antigenic specificity of IgG incomplete antibodies

In 1953, it was reported by Weiner et al., that auto-antibody in A.I.H.A. had blood group antigen specificity, in the case reported, an antigen from the Rhesus complex. Many reports have since confirmed, that in a significant number of cases, the auto-antibody in A.I.H.A. shows specificity for the Rh system (Dacie, 1962), often in the company of antibodies of undetermined specificity. The most important confirmatory observation is selective non-reactivity with rare, human Rh-null cells, which lack all Rh-antigens (Weiner and Vos, 1963).

Knowledge of the antigenic determinants with which such antibodies react is however incomplete. Leddy et al. (1970) found that only 12, from a total of 46 isolated auto-antibody preparations, did not react with Rh-null cells. The erythrocytes from these 12 patients had only IgG on their surface.
From/

From a further 20 patients with both IgG and complement on the red cell, 16 reacted strongly with Rh-null cells.

iii) Extravascular destruction of the erythrocyte

Recently, the study of macrophage and monocyte membrane receptors, has given some insight into the mode of destruction of IgG and complement sensitised erythrocytes in A.I.H.A.

Thus the interaction of antibody coated red cells is now known to be due to the presence of receptors for the Fc fragment of IgG, in particular of subclasses IgG1 and IgG3, on or within the membrane of the monocyte (Lo Buglio et al., 1967; Huber and Fudenberg, 1968; Abramson et al., 1970).

In addition, monocytes have been shown to have a receptor for C3 (Huber et al., 1968; Huber and Fudenberg, 1970), which has recently been shown to be, in mice, specifically for C3b (Griffin et al., 1975).

The process of removal of red cells, opsonised by either immunoglobulin or complement, in A.I.H.A., is thought to be predominantly by phagocytes, which form part of the newly defined mononuclear phagocyte system (Van Furth et al., 1975), and is thought to be due to the possession of Fc and C3 receptors by the phagocytic cells.

3. Prelude to the Work in this Thesis

In contrast to the large body of information which has accumulated regarding the final common pathway of red cell destruction, little is known of the early processes which operate in auto-immune haemolytic anaemia.

Thus it is not known why in A.I.H.A., immunocompetent cells should produce auto-antibodies against antigenic determinants on the patients' own red cells, despite many theories. Further, unlike a normal immune response which terminates spontaneously after a finite period, in many, but not all patients with A.I.H.A., the disorder is not self limiting.

This lack of understanding of both initiation and perpetuation persists, despite many years of practical research and of recent rapid progress in cellular immunology.

The major part of the work in this thesis is concerned with a phenomenon first noted in this laboratory, in patients with clinically active auto-immune haemolytic anaemia (Dewar et al., 1974). It was a cellular abnormality, recognised because of the increased affinity of certain peripheral blood mononuclear cells for homologous and autologous erythrocytes. The affinity was expressed in/

in vitro in the formation of rosettes, the latter characteristically being composed of a central mononuclear cell surrounded by attached erythrocytes. Rosettes formed with donor (homologous) erythrocytes have been called homologous rosettes, those with 'self erythrocytes' are called autologous rosettes.

The investigations, presented in Chapters III and IV, are predominantly studies of those rosetting cells in patients with auto-immune haemolytic anaemia.

Supplementary information is derived from investigation of other human disorders and in vitro experiments with mononuclear cells. The results of these investigations are discussed, with regard to identification of the central rosetting leukocyte and the part such cells might play in the cellular pathology of auto-immune haemolytic anaemia.

Despite the lack of knowledge concerning the aetiology of auto-immune haemolytic anaemia, treatment at present benefits a large number of patients suffering from this disorder. The most commonly used medication is corticosteroid.

Many of the patients studied in this thesis received this form of therapy. The last section of the thesis is concerned with experimental work designed/

designed to ascertain the changes in lymphocyte subpopulations which follow corticosteroid therapy in mice. In addition, the effect on in vivo and in vitro macrophage function by similar therapy is reported.

These changes are then related to the effect of corticosteroid on the clearance of IgG sensitised erythrocytes.

Each chapter contains its own review of the relevant literature.

CHAPTER II

MATERIALS AND METHODS

A. Human

i) Subjects

- a) Auto-Immune Haemolytic Anaemia (A.I.H.A.)
- b) Infectious Mononucleosis.
- c) Patients with miscellaneous disorders.
- d) Controls.

ii) Materials

- a) Ficoll-Hypaque
- b) Sheep red blood cells.
- c) Medium 199.
- d) Phosphate buffered saline.
- e) Ethylene diamine tetra-acetic acid.
- f) Neutral red.
- g) Trypan blue.
- h) Phytohaemagglutinin.
- i) Ferritin labelled antibody.
- j) Fluorescent antisera.
- k) Hepes buffer.

iii) Biological Materials, Human and Bovine

- a) Human red blood cells.
- b) Thymus tissue.
- c) Spleens - Lymph nodes.
- d) Human globulin.
- e)/

- e) Bovine serum albumin
- f) Rh-null erythrocytes

iv) Methods

- a) Preparation of homologous, autologous and sheep 'conventional' rosettes.
Peripheral Blood.
- b) Rosette preparation - Sheep 'Wigzell'.
- c) Preparation of rosettes from spleen, thymus, lymph node.
- d) Preparation of mixed rosettes
- e) Direct and indirect immunofluorescence
- f) Mixed fluorescence and rosetting
- g) Fluorescence with aggregated IgG
- h) Fluorescein conjugation
- i) Deaggregation
- j) Cytocentrifugation
- k) Direct and indirect antiglobulin testing
- l) Viability - Trypan blue
- m) Neutral red ingestion
- n) Electron microscopy - transmission
- o) Electron microscopy - ferritin-labelling
- p) Scanning electron microscopy
- q) Phytohaemagglutinin stimulation
- r) Glutaraldehyde fixation of rosettes

A. Human

i) Subjects

a) A.I.H.A.

Fourteen patients, with auto-immune haemolytic anaemia, were studied progressively for periods up to two years. Nine were female and five male. Their ages ranged from 11 - 82 years. A further eleven patients were investigated on a limited basis, in this latter group the disease was clinically quiescent.

The diagnosis in all cases depended upon the demonstration of a positive direct antiglobulin test, with a minimum titre of 1/40. The clinical activity of the disease was indicated by the following investigations and features: degree of anaemia, level of reticulocytosis and morphological evidence of active haemolysis, such as spherocytosis and fragmentation of erythrocytes in the blood film.

Of the patients followed serially, only one, a female of 36 (I.W.), had an underlying disorder, this was disseminated lupus erythematosus. Two of the patients investigated to a limited extent had underlying disease, a male aged 52 (F.K.) with scleroderma, and a male aged 73 (R.S.) with chronic lymphocytic leukaemia.

Most patients were on oral steroids at varying stages/

stages and in varying dosages. Some patients had to undergo splenectomy to control the disorder. Only two, T.K. aged 11 and R.G. aged 86, required Azathioprine in addition to steroid therapy.

b) Infectious Mononucleosis

Eight patients with this disorder were investigated (4 male - 4 female). The age range was 18 - 26 years. The diagnosis, suspected clinically because of lymph node enlargement with accompanying sore throat, was confirmed by standard laboratory investigations. These were the demonstration of atypical lymphocytes on the blood film, and a positive adsorbed Paul-Bunnell reaction. Three patients were studied on three or four separate occasions, two of these received steroid therapy. None of the other subjects received specific therapy.

c) Patients with miscellaneous disorders

This was a group of patients with disorders of the red cell; hereditary spherocytosis, paroxysmal nocturnal haemoglobinuria, hexokinase deficiency. Some had haemolysis due to artificial heart valve replacement, methyl-dopa therapy and sideroblastic anaemia. Patients studied with white cell disorders were suffering from plasma cell/

cell leukaemia, acute lymphoblastic leukaemia, chronic lymphocytic leukaemia, two patients with an idiopathic monocytosis and one with an idiopathic lymphocytosis.

d) Controls

Three control groups were studied:

1. Laboratory personnel (11).
2. Hospital in-patients with miscellaneous non-haematological disorders (18).
3. Elderly in-patients with a lower age limit of 70 (10).

ii) Materials

a) Ficoll-Hypaque

Ten parts of 75.3% Hypaque (Winthrop) were mixed with 24 parts of Ficoll (Pharmacia). The mixture was sterilised by membrane filtration and stored in 10 ml aliquots at room temperature, in sterile universal containers.

b) Sheep Red Blood Cells

These were supplied from Moredun (Agricultural Research Unit, Edinburgh). As few different sheep as possible were used. 20 ml of blood was obtained by venesection and mixed with 5 ml of Alsever's solution which contained; 100 Iu/ml of penicillin and 200 ug/ml of streptomycin sulphate. The erythrocytes were stored at 4°C and/

and were used within two weeks. Before use they were washed three times in phosphate buffered saline (P.B.S.).

c) Medium 199 (Wellcome Laboratories)

After the addition of 7.5 ml of 4.4% NaHCO_3 to 100 ml of 10x 199, the whole was made up to one litre with distilled water, and 200,000 units of penicillin and 100,000 units of streptomycin added.

Ten units/ml of heparin were added when heparinised 199 was used. For 199 with HEPES Buffer 0.48 g HEPES Buffer was added to 100 ml of medium.

The medium was stored in 100 ml aliquots at 4°C .

d) Phosphate Buffered Saline (P.B.S.)

The initial solution contained 85 g NaCl , 10.7 G $\text{Na}_2 \text{HPO}_4$ and 3.9 G $\text{Na H}_2 \text{PO}_4$ per litre. This was then diluted x 10 with distilled water and stored at room temperature in 100 ml aliquots.

e) Ethylene-diamine tetra-acetic acid

(E.D.T.A.).

This was made by dissolving 2 G of EDTA in 100 ml of distilled water. The resulting solution was sterilised through a millipore filter.

f/

f) Neutral Red

This was a 0.1% solution in
9g/l NaCl.

g) Trypan Blue

This was made up as a 2% solution
in distilled water.

h) Phytohaemagglutinin

This was obtained from Burroughs-
Wellcome (reagent grade).

i) Ferritin Labelled antibody

This was goat, anti-human IgG-IgA-
IgM, labelled with ferritin, and was obtained from
Cappel Laboratories (Downington P.A.).


j) Fluorescent antisera

Commercially available (Nordic)
*FITC labelled antisera were used.

k) Hepes Buffer

This was supplied by Flow
laboratories (U.K.).

iii) Biological Material, Human and Bovinea) Human Red Blood Cells

Outdated blood of various groups
was obtained from Edinburgh Blood Transfusion
Service. It had been stored at 4°C in  for
a period exceeding three weeks.

*fluorescein isothiocyanate.

b) Thymus Tissue

These organs were either obtained from patients undergoing cardiac bypass, or post mortem.

c) Spleens - Lymph Nodes

These tissues, when studied, were obtained directly from the operating theatre and processed immediately.

d) Human Globulin

This was obtained from the Blood Transfusion Service Edinburgh.

e) Bovine Serum Albumin (B.S.A.)

This was supplied as a 35% sterile solution by Sigma (London).

f) Rh-null erythrocytes

Dr. S. Worlledge very kindly supplied these cells, which had been stored in liquid nitrogen. Control frozen erythrocytes were provided by Dr. D. Pepper (Edinburgh, Blood Transfusion Service).

iv) Methods

a)/

a) Preparation of Homologous, Autologous
and Sheep 'Conventional' Rosettes
Peripheral Blood, Mononuclear Cell
Separation

In most cases, 20 ml of venous blood was taken into 2 ml of 2% E.D.T.A. in a glass Universal bottle. 10 ml aliquots of this anti-coagulated blood were carefully layered onto Ficoll/Hypaque mixture and then centrifuged for 20 minutes at 20°C in standard siliconised universals (500 g at the blood/ficoll interface). The resulting leucocyte band was carefully removed by Pasteur pipette and placed in plastic tubes containing 10 ml medium 199. The cells were washed three times in a large excess of this medium (200 g for 5 min.) and then resuspended. In a majority of cases, white cell counts were carried out, prior to, and following the procedure, and the respective volumes measured. Mononuclear recovery could then be calculated. In A.I.H.A., glandular fever and controls this was on average one third of the starting mononuclear population. Trypan Blue viability was assessed on initial samples following separation. In later specimens this procedure was not carried out.

Red Blood Cells

Autologous red blood cells and homologous red blood cells (from an O-negative member/

member of staff) were washed three times in P.B.S. They were then resuspended in P.B.S. and counted. Cells (stored at 4°C) which were older than three days were discarded.

Rosette preparation

40 x 10⁶ red blood cells

(autologous, homologous or sheep red cell) were added to 1 x 10⁶ of the mononuclear cells under investigation in a siliconised Wasserman tube. The volume was made up to 1 ml with Medium 199. After incubation for 10 minutes at room temperature the cells were pelleted by centrifugation (200 g for 5 min.) and then left overnight at 4°C.

Counting of Rosettes

The cells were resuspended by very gentle inversion (x 5) of the tube. An amount sufficient to fill each chamber was placed by Pasteur pipette on each side of a haemocytometer (Improved Neubauer). The preparation was overlaid with a cover-slip and the cells allowed to settle. Examination was by light microscope at a magnification of 400 X. Rosettes (white cells having attached to them three or more red cells) were counted on both sides of the chamber and in duplicate. At least 8 sq. mm. were counted. The white count of the preparation was then determined and/

and the percentage of rosettes calculated using the following formula:

$$\% \text{ rosettes} = \frac{\text{Number of Rosettes}}{\text{Number of sq.mm. counted}} \times \frac{1}{\text{W.B.C.} \times 10^6}$$

Alternatively the number of rosettes in a minimum of 400 white cells were counted.

Variations on the mode of preparation of these rosettes; temperature, medium supplements and changes in erythrocyte/white cell ratio, will be alluded to in the relevant sections in the results.

b) Rosette preparation - Sheep 'Wigzell' -
To quantitate T lymphocytes

40 x 10⁶ sheep erythrocytes with 1 x 10⁶ white cells were incubated at 37°C for 5 minutes. It was then spun at 800 g for 5 minutes and incubated for a further 2 hours at 4°C.

The cells were then resuspended by gentle rotation. A drop was placed on each side of a counting chamber by Pasteur pipette. The rosettes in a minimum of 200 white cells, in duplicate, were enumerated. (A rosette being defined as a white cell with a minimum of three red cells attached).

c) Preparation of Rosettes from - Spleen
- Thymus - Lymph node

A small piece of tissue was removed from/

from each specimen by scalpel. The portion was then gently teased apart in ice cold medium 199, using either fine forceps or two hypodermic needles. Large aggregates were allowed to settle, the supernatant cells were then removed and washed three times in large volumes of heparinised 199. In the case of the spleen, if red cell contamination was marked, a preliminary separation on ficoll/hypaque was performed. The white cells were then counted.

The subsequent setting up and quantitation of homologous and autologous rosettes was as previously described for peripheral blood.

d) Preparation of Mixed Rosettes

These were of either mixed sheep and autologous erythrocytes or sheep and homologous erythrocytes. 20×10^6 homologous or autologous red blood cells were added to 1×10^6 white cells, followed five minutes later by 20×10^6 washed sheep red cells. Each preparation was made up to 1 ml with 199 if required. The rosette procedure as described was repeated. The quantitation of rosettes (any white cell in contact with a minimum of three red cells of any type) was on 200 cells in separate samples, and performed in duplicate. Cells were scored as; (1) non-rosetting, (2) sheep cell rosetting, (3) sheep and homologous (autologous) rosetting, (4) homologous (autologous) rosetting.

e) Immunofluorescence (To detect B lymphocytes)

Both direct and indirect techniques were used. (F.I.T.C. - fluorescein isothiocyanate).

1) Direct fluorescence

3×10^6 white cells from the sample under study were placed in a siliconised Wasserman tube. 0.05 ml of deaggregated, swine anti-human IgM - IgA - IgG, F.I.T.C. conjugated antibody, was added and the volume made up to 1 ml with P.B.S. The cells were incubated for 30 minutes at 4°C then washed twice (200 g for 5 minutes) in P.B.S. They were resuspended in a small volume, a drop removed and placed on a glass slide, and sealed under a coverslip. Examination and quantitation was as described in the next section.

2) Indirect Technique

0.1 ml of rabbit, anti-human IgM - IgA - IgG antibody was added to 2×10^6 washed white cells in 0.9 ml P.B.S., the cells evenly suspended and incubated for 10 minutes, at 37°C , in siliconised Wasserman tubes. The cells were washed twice (200 g/5 minutes) in P.B.S. and cooled to 0°C in melting ice. To the cell suspension was added a 1/20 dilution of goat anti-rabbit serum (F.I.T.C.)* and incubation was continued for a further 10 minutes. The cells were/

*0.05ml.

were then washed twice in P.B.S., resuspended in a small volume, and a drop placed on a slide and sealed under a coverslip with nail varnish. Slides were examined immediately.

Examination was by a Leitz Ortholux research microscope, with an HBO 200 mercury vapour lamp, BG38 and BG12 excitation filters and a K510 suppression filter.

Conventional illumination was employed alternately with fluorescence for cell counting and morphological assessment. A minimum of 100 cells were evaluated.

f) Mixed Fluorescence and Rosetting

The direct fluorescent technique was performed and 40×10^6 erythrocytes, of the required type, were added to the resultant white cells.

Appropriate rosettes were then prepared as previously described. These preparations were quantitated 3 hours after incubation at 4°C . The cells were gently resuspended, and a drop of the suspension placed on a glass slide and sealed on a coverslip as before. At least 200 cells were evaluated for rosetting and fluorescence.

g) Fluorescence with aggregated IgG

This was carried out by the method
of/

of Dickler and Kunkel (1972). Briefly, human globulin, at a concentration of 50 mg/ml in P.B.S., was heat inactivated at 56°C for 30 minutes and then conjugated with fluorescein. Conjugated preparations were heat aggregated at 63°C for 15 minutes, pelleted, and homogenised in P.B.S. Just before use aggregates were spun at 1000 g for 30 minutes and the concentration adjusted to approximately 3 mg/ml. A direct technique was used. 0.05 ml of the F.I.T.C. aggregated IgG was added to 1×10^6 leukocytes suspended in 0.2 ml of P.B.S. They were incubated at 4°C for 30 minutes and then washed twice in P.B.S.. Cells were prepared for fluorescent examination as before.

In many cases this procedure was combined with rosetting.

h) Fluorescein Conjugation

This was carried out as described by Nairn (1969).

i) Deaggregation

The relevant F.I.T.C. conjugated antisera were spun, at 150,000 g for 30 minutes, in an M.S.E. superspeed centrifuge before use.

j) Cytocentrifugation

In preparations where this was carried/

carried out the following procedure was used. The rosette preparation was gently resuspended and diluted x 10 with P.B.S. 0.5 ml of this was then spun on a Shandon-Elliott cytocentrifuge at 100 revolutions per minute for 8 minutes. The slides were removed, air dried and stained with Giemsa.

k) Direct and Indirect Antiglobulin

Testing

1) Direct

Erythrocytes, taken from the patients clotted blood, were washed x 4 in 0.9 m saline. These cells were then tested against 1/10, 1/20, 1/40 and 1/80 dilutions of a polyvalent human, antiglobulin reagent (West of Scotland B.T.S. Service) and the tests read microscopically. Positive tests were then reread using monospecific antiglobulin (anti-IgG, anti-IgM and anti-IgA) and antisera to ~~complement~~* at each anti-human globulin's optimum dilution. For positive results, a series of dilutions of that particular monospecific anti-human globulin was prepared, and the cells retested. The antiglobulin titre was expressed as the weakest dilution of monospecific anti-human globulin causing microscopic agglutination.

2) Indirect

The patient's serum was incubated with/

*(C3)

with washed, standard erythrocytes at 37°C for 60 minutes. The erythrocytes were then washed x 4 in 0.9% saline, and tested on a slide, using a polyvalent, human anti-globulin reagent, diluted to its optimum for the detection of IgG and complement coating. Tests were read microscopically.

1) Viability - Trypan Blue

2% Trypan blue was diluted to 1% with P.B.S. 0.1 ml of Trypan blue and 0.1 ml of cell suspension were mixed on a glass slide. Viability was expressed as the percentage of cells excluding the dye and in all cases tested exceeded 90%.

m) Neutral Red Ingestion (To quantitate monocytes)

0.1 ml. of a white cell suspension under investigation, was placed in 1 ml of 199 containing 0.1 ml of neutral red. The whole was incubated at 37°C for 15 minutes, then centrifuged (400 g for 5 min.) and the supernatant poured off. The percentage of cells ingesting neutral red was then counted.

n) Electron Microscopy

Preparations were examined from ten patients with A.I.H.A. (some of these on several occasions during the course of the disease) and four with glandular fever.

Autologous/

Autologous and homologous rosette preparations were set up in parallel when required for electron microscopy. The technique of preparation was similar, apart from reducing the number of red cells added to 10×10^6 . Following incubation at 4°C , the cells were not resuspended, but the medium removed and the pellet fixed in 1.5% ice cold glutaraldehyde (pH 7.4) for 15 minutes. The pellet was then washed in 0.1 M cacodylate buffer (pH 7.4), post-fixed in osmium tetroxide (1%) in cacodylate buffer for 10 minutes, then washed again in buffer. The specimen was dehydrated through alcohols then embedded in araldite in gelatin capsules. Sections (50 nanometres thick) were cut on an ultra microtome (Reichert OMU3), placed on copper grids, then stained with lead citrate and uranyl acetate. The sections were examined and photographed on a Corinth 275 AEI microscope.

o) Electron Microscopy - Ferritin labelling

A direct technique was used.

Peripheral blood cells were separated and washed as for rosette preparation. 20×10^6 white cells, in 2 ml of 199, were incubated with 0.5 ml of ferritin conjugated, goat anti-human IgM - IgA - IgG globulin, at 4°C for 45 minutes. The cells were washed in large volumes of P.B.S. three times over a period of 30 minutes, at 4°C . The rosettes were prepared by adding 10×10^6 homologous erythrocytes and incubation carried out overnight at/

at 4°C. The supernatant was removed and the pellet initially fixed in 4% glutaraldehyde, post-fixed and dehydrated as previously described, and finally stained with Bismuth. Examination again was on a Corinth 275 AEI microscope.

p) Scanning Electron Microscopy

Homologous and sheep cell rosettes (conventional) were prepared as described, but with only a 20 : 1 erythrocyte/leukocyte ratio. After gentle resuspension a drop was placed on a small glass coverslip. The cells were allowed to settle onto the glass for 15 minutes. Following that, 0.2 ml of 1% osmic acid was added and fixation allowed to proceed for 30 minutes. The coverslips were then taken, without prior washing, through a graded series of acetone (10 - 30 - 50 - 70 - 90 - 100%) and were kept in each for at least 2 minutes. Care was taken to avoid drying of the coverslip. Storage was then in 100% acetone and silica gel until further processing could be carried out.

The final steps were critical point drying from liquid CO_2 in a critical point drier (Polaron), the coverslips were then mounted on an aluminium stub and given a 500 Å gold coating in a Polaron Diode sputterer. The specimens were then/

then examined and photographed by means of a Cambridge S180 Scanning Electron Microscope (By courtesy of the Medical Research Council, Population and Cytogenetics Unit, Western General Hospital, Edinburgh).

q) Phytohaemagglutinin Stimulation

Blood was collected, either from volunteers (100 - 250 ml), or patients (50 ml), into E.D.T.A. (40 mg/100 ml blood). The mononuclear cells were then separated aseptically by differential gradient centrifugation on Ficoll/Hypaque as described, and were washed three times in 199. They were then made up to a concentration of 0.5×10^6 /ml in 199, containing Hepes buffer and 15% blood group compatible serum (heat inactivated). 2 ml was placed in round bottomed test tubes and 10 μ l PHA was added to each of half of the tubes, the others acting as controls. The cells were incubated for 72 hours at 37°C. At required intervals test and control tubes were removed. The cells were washed 3 times in P.B.S. and then homologous, autologous and in some cases sheep cell rosettes were set up and quantitated as described.

r)/

r) Glutaraldehyde fixation of rosettes

This was carried out on several occasions in the autologous and homologous preparations. Within 10 seconds after resuspension glutaraldehyde was added to achieve an overall concentration of 0.6%. The rosettes were left for 20 minutes at 4°C to fix fully then diluted with distilled water, centrifuged and resuspended. The rosettes were then examined with particular regard to the number of erythrocytes attached to the R.F.C. This method is that of Haskill et al. (1972).

B. MICE

i) Strains Used

ii) Materials

- a) AKR anti C3H serum (anti- θ serum)
- b) Fluorescent antisera
- c) Cortisone acetate
- d) Rabbit anti-mouse red blood cell serum
- e) Other reagents.

iii) Methods

- a) Regimens of cortisone acetate used
in vivo for cell population changes.
- b) Lymphoid population changes in spleen,
thymus and lymph nodes.
- c) Red cell clearance studies in
Schofield mice.
- d) The effect of in vivo and in vitro
cortisone acetate on the phagocytic
ability of mouse peritoneal monolayers.
- e) Preparation of sensitised sheep red
cells.

B. Mice

i) Strains used

C3H mice (Banting and Kingman, Hull, England), within the weight range 18 - 28G, were used for the studies to evaluate cellular changes due to therapy with cortisone acetate.

Schofield mice (Department of Physiology, University of Edinburgh) were used for erythrocyte clearance studies and the investigations of in vivo and in vitro effects of steroids on peritoneal macrophages.

ii) Materials

a) AKR anti-C3H serum (Anti- θ serum)

This was obtained from G.D. Searle (High Wycombe, England). The injection schedule for production of this antiserum was introduction of C3H thymus cells, for 8 weekly injections, into the peritoneal cavity of AKR mice. Seven days after the final injection blood was harvested from the AKR mice by cardiac puncture. It was tested against most of the known C3H locus positive strains and was additionally shown to be negative for AKR thymus cells. The serum was maximally active to a dilution of 1/200. In the investigation here it was first deaggregated by centrifugation, diluted x 10 in P.B.S. and stored in 0.1 ml aliquots at -20°C .

b)/

b) Fluorescent antisera

F.I.T.C., polyvalent swine anti-mouse immunoglobulin or goat anti-mouse immunoglobulin were obtained from Nordic pharmaceuticals.

c) Cortisone Acetate

This was obtained from The Boots Company, Nottingham, England, in the form of Cortistab (25mg/ml).

d) Rabbit anti-mouse red blood cell serum

This was obtained from Cappel Laboratories (Downington, P.A.).

e) Other reagents

Medium 199, Bovine Serum Albumin, Trypan Blue and Ficoll-Hypaque were used as described for the human experiments.

iii) Methodsa) Regimens of cortisone acetate used in vivo for cell population changes

Two regimens of Cortisone acetate were used:

1. One group received 6mg of cortisone acetate intraperitoneally and no further medication.

2. The second group were given, by the same route, 6mg of cortisone acetate and 1.5mg daily thereafter. Control mice received 0.1ml/

0.1ml of P.B.S. corresponding to each injection.

Both groups were studied at various intervals following injection. Mice were killed by cervical dislocation and the following studies were carried out:

b) Lymphoid population changes in spleen, thymus and lymph nodes

These organs were removed and weighed.

In the case of the thymus care was taken to remove adherent lymph nodes. Lymph nodes for study were removed from the axillae, the inguinal regions and the para-aortic region. All organs, once removed and weighed, were placed in ice cold 199 containing B.S.A. (final concentration 1%). They were then separately homogenised in a known volume of 199. (containing B.S.A.), in a glass homogeniser with a loose fitting stopper. The nucleated cell count was then determined. The cells were washed three times in medium, the white cell count adjusted to 20×10^6 /ml (where possible) and 0.05ml aliquots placed in glass precipitin tubes. These were centrifuged (200g for 5 min.) and the supernatant removed, following which 0.05ml of anti-serum (Θ) was added to half the tubes and 0.05ml of medium 199 to the remainder. Incubation at 37°C for 30 minutes/

minutes was followed by washing three times in P.B.S.. After the final wash the pellet of cells was left at the bottom of the tube, and in control and test, was resuspended in 0.05ml of neat, goat or swine (depending on availability) polyvalent anti-mouse Ig (FITC). The tubes were incubated for 15 minutes at room temperature and washed twice in P.B.S.. A drop was placed on a slide and sealed under a coverslip. Counting of the specimens was carried out within 2 hours, and the slides prior to inspection were kept at 4°C.

Initially an attempt was made to identify T and B lymphocytes by their pattern of surface fluorescence as described by Raff (1970). This author described T lymphocytes as having a circular pattern of fluorescence and being easily distinguished from B lymphocytes which had a characteristic capping appearance. This capping form of fluorescence is in fact illustrated in Fig. XXI (Chapter III) and 'ring' fluorescence is illustrated below.

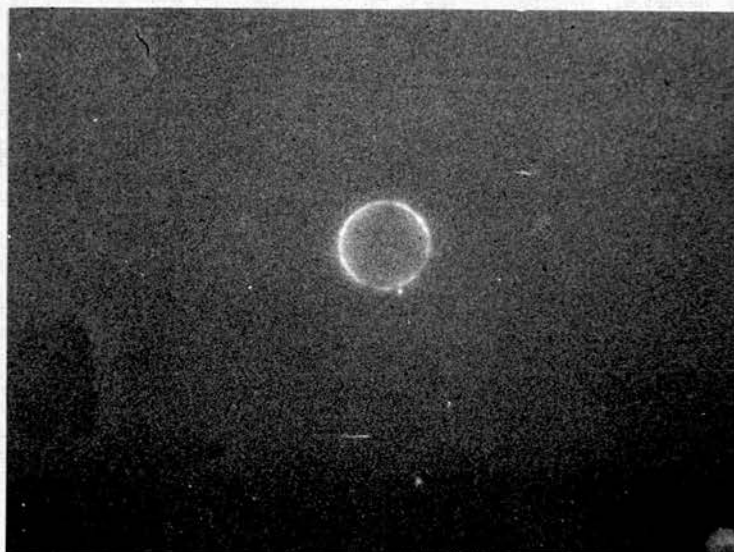


FIGURE Circular pattern of fluorescence encountered in the lymphocyte preparations and said to be characteristic for T-lymphocytes (Raff, 1970).

Such morphological differences were identified in the preparations but did not reliably estimate T and B lymphocyte numbers.

For the final figures therefore, T and B lymphocytes have been calculated as follows:-

B lymphocytes = percentage of fluorescent cells following treatment with anti-mouse immunoglobulin (F.I.T.C.) only.

T lymphocytes = percentage of fluorescent cells following treatment with anti- Θ serum and anti-mouse immunoglobulin (F.I.T.C.)^{*} - percentage of fluorescent cells following treatment with anti-mouse immunoglobulin (F.I.T.C.) only.

*minus

c)/

c) Red cell clearance studies in
Schofield Mice

Test animals received 6mg cortisone acetate three days prior to the experiment. Red cells were removed from the mice by retro-orbital puncture and washed three times in P.B.S., then incubated for 1 hour at 37°C with the appropriate dilution (1/1000, 1/1250, 1/1500) of antiserum. The cells were then washed twice in P.B.S. and stored with a packed cell volume of 50% overnight at 4°C. The next morning the cells were incubated at room temperature for 30 minutes with ⁵¹Cr (10 uc Amersham, England), then washed three times in P.B.S. and made up to a 10% suspension. Control red cells were similarly treated but were not incubated with antiserum.

0.1ml of the appropriate red cell suspension was then injected into the tail vein of the mouse under study. 0.25ml of blood was taken from the retro-orbital sinus at appropriate time intervals by means of a calibrated pipette and put into 1ml of distilled water in plastic tubes. After the final blood sample the mice were killed by cervical dislocation and weighed. The liver and spleen were removed, weighed, and weighed samples of the organs were placed in plastic tubes. All samples were/

were then counted in a well type scintillation counter (Tracerlab I.C.N.).

The clearance index K, was calculated according to the following formula:

$$K = \frac{\text{logarithm of scintillation counts at T1} - \text{logarithm of scintillation counts at T2}}{T2 - T1}$$

$$T2 - T1$$

Where T1 = Time 1

T2 = Time 2

d) The effect of in vivo and in vitro
cortisone acetate on the phagocytic
ability of mouse peritoneal monolayers

Schofield mice were given intraperitoneal cortisone acetate for three days. The daily dosage levels were 5mg; 0.5mg; or 0.05mg. Controls received intraperitoneal saline. Macrophages from the peritoneal cavity were harvested by flushing with 3-5ml of 199 per mouse and made up to a final white cell concentration of 1×10^6 /ml in 199 containing 10% human serum (sterile, heat inactivated, blood group AB +ve). 1ml of this suspension was put into glass test tubes with 'flying coverslips' and were then incubated for 5-6 hours at 37°C. To some tubes after/

after this period cortisone was added at levels of 2.5mg, 0.25mg or 0.025mg. The cultures were incubated a further 18 hours at 37°C.

At this point to half the tubes was added 0.1ml of a 5% solution of sheep red blood cells (S.R.B.C.). To the other half, a similar number of IgG-sensitised S.R.B.C. was added.

Incubation, for a further two hours, was terminated by washing the coverslips twice in P.B.S., fixing them in methanol for 10 minutes and then staining with giemsa. The number of macrophages ingesting erythrocytes were then counted in a minimum of 500 cells, by two observers in duplicate.

e) Preparation of sensitised sheep red cells

Washed sheep cells were incubated in 7S, rabbit-anti-sheep, red blood cell serum, in a concentration equivalent to 80% of the minimum agglutinating titre for 1 hour at 37°C. Sensitised cells were then washed twice in P.B.S. and resuspended at a 5% concentration.

CHAPTER III

CLINICAL AND EXPERIMENTAL
STUDIES OF HOMOLOGOUS AND
AUTOLOGOUS ROSETTING CELLS

1. Background
2. Antigen sensitive cells in animals
 - i) Rosetting techniques
 - ii) Conclusions
3. Heterologous-erythrocytic reactions in man
 - i) Rosetting techniques
 - ii) Conclusions
4. Homologous and autologous erythrocytic interactions with lymphocytes in animals.
 - i) Interactions in animals.
 - a) Rabbit
 - b) Mouse
 - ii) Conclusions
5. Autologous and homologous rosetting reactions in man.
6. Aims of the present investigation.

1. Background

Experimental techniques which depend upon the formation of a rosette, the latter recognised as a cluster of erythrocytes around a central mononuclear cell, are now widely applied in immunology. They were first formally described by Zaalberg (1964), who studied spleen cells from mice previously immunised with sheep red blood cells (S.R.B.C.). S.R.B.C. were used subsequently to detect specific antibody-forming cells. As used in this system, rosettes formed, are specific for the immunising antigen (Shearer and Cudkowitch, 1968).

Rosette formation dependent on such specific immunisation has been described in man. Thus, in pregnant Rh-isoimmunised patients and also in volunteer male donors (Rh-negative) injected with Rh-positive cells, peripheral blood leukocytes form rosettes with Rh-positive but not with Rh-negative erythrocytes (Elson and Bradley, 1970). These rosetting leukocytes are probably actively immunised to the Rh-antigen.

Initial experiments, carried out in this laboratory, to detect circulating antibody producing cells in auto-immune haemolytic anaemia (A.I.H.A.) by the plaque technique (Jerne and Nordin, 1963) failed./

failed. An attempt was then made to identify circulating antibody producing cells by rosette formation. Auto-antibodies in A.I.H.A., as explained in Chapter I, often show specificity for the Rh complex. Thus the cells producing antibody should be detectable using normal human erythrocytes.

Gluckman (1970) reported briefly that the peripheral blood, from patients with A.I.H.A., showed an increase above control subjects, of leukocytes forming rosettes with autologous erythrocytes. Criticism was levelled at this report because autologous erythrocytes in A.I.H.A. are sensitised with IgG and/or complement in most cases (Hamblin and Verrier-Jones, 1970). As receptors for IgG and complement are found on both circulating monocytes and B-lymphocytes, difficulties in interpretation result (Huber and Fudenberg, 1970; Fröland and Natvig, 1973).

To circumvent such criticism, the experiments in this laboratory utilised, in addition to autologous erythrocytes, homologous red blood cells. The latter are unsensitised. Preliminary results in A.I.H.A. showed that the numbers of homologous-rosetting cells were raised when compared with control subjects.

It/

It was difficult to avoid the conclusion that such cells played a part in the cellular pathology of the immunological disturbance underlying A.I.H.A.. It was decided that a detailed study of these cells might provide insight to the underlying mechanisms which initiate and perpetuate this disorder.

Initial inhibition experiments with anti-human-globulin sera were unsuccessful and testing for antigen specificity was not feasible because Rh-null cells were not available. At this stage the nature of the rosetting cell was in doubt but there appeared to be three possibilities. The rosette forming cell (R.F.C.) might be immune and this aspect has been discussed in the opening paragraphs of this chapter. Immune-R.F.C. are predominantly B-lymphocytes, though Bankhurst and Wilson (1971) have provided evidence that a proportion are T-lymphocytes.

Two other major classes of cell could account for such rosetting reactions. The first possibility was that antigen-sensitive cells were responsible. The second possibility was that the interaction was non-specific and similar to autologous and homologous lymphocytic interactions described in animals and more recently in man.

These two forms of interaction and the type of lymphocyte (T and B) responsible for them will now be/

be reviewed.

2. Antigen-Sensitive Cells in Animals

These can be either T or B lymphocytes. Their presence in cell suspensions has been revealed in non-immunised animals by rosetting with particulate heterologous antigens such as S.R.B.C.

i) Rosetting techniques

Many groups of workers have identified cells which rosette with S.R.B.C. in spleen cell suspensions from unimmunised animals (Biozzi et al., 1966; Biozzi et al., 1968; Laskov, 1968; McConnell et al., 1969; Bach et al., 1970). According to these workers such cells include macrophages, natural antibody producing cells and antigen sensitive cells.

That antigen sensitive cells contribute significantly to these populations has been shown elegantly by Bach et al. (1970). Depletion of S.R.B.C. rosetting cells can be carried out by differential centrifugation. Subsequent injection of such specifically depleted populations into cyclophosphamide treated mice results in a markedly diminished antibody response to the relevant antigen, in this particular case S.R.B.C.

Some/

Some of these antigen sensitive cells are B cells, having been demonstrated in normal and neonatally thymectomised mice (15 days after birth) and some bear Ig receptors (Biozzi et al., 1969; Hogg and Greaves, 1972). Haskill et al. (1972) have presented strong evidence that S.R.B.C. rosetting cells, in non-immunised mice, are a composite of T and B cells.

Evidence has been presented recently by one group of workers that just over one third of these cells in mice are thymus derived (Ashman and Raff, 1973). The experiment cited, involved the direct demonstration of Θ antigen, an antigen unique for mouse T cells (Reif and Allen, 1963), on S.R.B.C. rosetting cells.

Further evidence in support of a T cell contribution to S.R.B.C. rosetting cells in mice, albeit indirect, has been provided by other authors.

Thus the number of rosettes from cell suspensions are reduced, following treatment with anti- Θ serum in the presence of complement, a procedure which kills T-lymphocytes. The sensitivity to anti- Θ of such cells from different sites varies, those of the thymus and spleen which recirculate rapidly/

rapidly are less sensitive to anti- θ serum (Bach and Dardenne, 1973). This varying sensitivity to anti- θ serum is perhaps explained by the differing amount of θ -alloantigen on T cells, corresponding to such a pattern of sensitivity (Cantor, 1972; Stobo and Paul, 1973).

The results of experiments using anti- θ serum should be interpreted with caution, as such antisera contain anti-allotype and auto-antibodies (Greaves and Raff, 1971).

In addition to experiments employing anti- θ serum, it has been shown that in the thymus, S.R.B.C. rosetting cells reside in the hydrocortisone resistant medullary population (Bach and Dardenne, 1972). The hydrocortisone resistant cells in the thymus have many of the attributes of peripheral T cells (Greaves et al., 1974a). In addition, thymectomy in mice is followed within 5 - 6 days by a fall in splenic S.R.B.C. rosetting cells (Bach, 1973a).

The debate concerning the existence of a thymic contribution to such rosetting cells in non-immunised mice is of relevance, as much information derives from rosette techniques.

ii) Conclusions

These studies have shown that in non-immunised animals there exists a population of lymphocytes/

lymphocytes which will react specifically with heterologous antigens and which can be demonstrated using rosetting techniques. That such populations consist of both T and B lymphocytes is well established but the exact contribution made by each to the total is unknown. The exact function of such cell populations is at present unclear.

3. Heterologous-erythrocytic reactions in man

The application of rosetting techniques in man with various heterologous erythrocytes has, for reasons of accessibility, been mainly restricted to studies on blood.

i) Rosetting techniques

Though other xenogeneic erythrocytes have been used the most important observations have concerned lymphocytic interactions with S.R.B.C. Bach et al. (1969) were the first to report that a proportion of human lymphocytes formed rosettes with S.R.B.C., this finding was soon independently confirmed by three groups of workers (Coombs et al., 1970; Brain et al., 1970; Lay et al., 1971).

The first to suggest that such rosetting lymphocytes were thymus-derived were Wybran et al. (1972) though other workers almost simultaneously came/

came to the same conclusion (Fröland, 1972; Silveira et al., 1972). The receptor responsible for binding of sheep cells in this system has not been identified. At present, the adherence is attributed to a non-immune, non-specific phenomenon (Bach, 1973b).

ii) Conclusions

The human lymphocytes which form rosettes with S.R.B.C. all belong to the T cell population. The rosettes are not antigen specific. They are probably due to a differing mechanism than S.R.B.C. rosetting cells formed in the mouse, which are antigen specific.

4. Homologous and Autologous erythrocytic interactions with lymphocytes in animals

This chapter is concerned primarily with homologous and autologous red cell interaction with lymphocytes in A.I.H.A. Recent work describing similar interactions in animals, and in man in general, is now reviewed.

i) Interactions in animals

Autologous adherence between macrophage-thymocyte (Siegel, 1970a,b) and lymphocyte-Leydig cell (Rivenzon et al., 1974) has been described but most attention has been directed to the interaction/

interaction of lymphocytes with homologous or autologous red cells.

a) Rabbit

Siegel (1970c) reported that rabbit thymocytes adhered to both autologous and homologous red cells and noted that lymph node cells did not show similar affinities. There was no heterologous interaction with rat, mouse, guinea pig or human red cells. In a later paper three further characteristics were delineated (Siegel and Sherman, 1972); an increased number of rosettes was noted with newborn rabbit thymocytes, adherence occurred with nucleated red cells present in the bone marrow and pre-treatment of thymocytes with trypsin completely abolished autologous thymocyte-erythrocyte adherence. The latter finding indicating that the red cell receptors are part of, or are dependent upon the presence of surface protein molecules.

b) Mouse

In contrast to Laskov (1968) who found no autologous rosettes in mouse spleen cell suspensions, Micklem (1971) found that between 10^2 to 10^4 per 10^6 nucleated cells in the lymphoid tissue of normal mice formed rosettes. No difference in number was found between autologous and/

and homologous rosettes (called by the author auto-clusters and synclusters respectively). Such cells were highest in the thymus and similar levels were found in germ free mice. Evidence presented in a subsequent paper indicated that many of these R.F.C. were not T cells, but the actual proportion of T and B lymphocyte was not determined (Micklem and Anderson, 1972).

Charrière and Bach (1974) have presented evidence to show that the numbers of autologous rosette forming cells are highly thymus dependent. Thus following thymectomy, the percentage of autologous R.F.C. increased 20 fold and similar high levels of autologous R.F.C. were found in six-week old nude mice (athymic) and in four month old New Zealand Black mice (a strain which develops auto-immune disease).

ii) Conclusions

In rabbits and in mice, autologous and homologous interaction can be demonstrated between lymphocytes and red cells. There is controversy as to the contribution of T and B cell to such lymphocytic populations. The nature of the receptor responsible for adherence has not been characterised.

The numbers of autologous rosettes are low in normal mice but are increased in New Zealand Black/

Black mice which are known to develop auto-immune haemolytic anaemia.

5. Autologous and Homologous rosetting reactions
in man

Since the investigations in this thesis were initiated there have been a number of publications describing autologous and homologous rosette formation in man. As they have, to some extent, dictated the final direction of the experiments given here they will be reviewed now.

Though previous workers (Lay et al., 1971; Silveira et al., 1972) using either lymphocytes in suspension or in the form of tissue sections did not find lymphocytic interaction with autologous human red cells, Baxley et al. (1973) reported binding of human erythrocytes to human thymocytes. Tonsil and peripheral blood lymphocytes gave negative reactions unless treated with neuraminidase.

Following the latter report peripheral blood autologous rosetting lymphocytes have been reported by many workers (Charrière and Bach, 1974; Sandilands et al., 1974).

Kaplan (1975) reported that peripheral blood lymphocytes formed rosettes with homologous erythrocytes and recorded levels of up to 6%
in/

in normal subjects.

Recent evidence, from normal controls and from patients with clinical disorders involving increased circulating T lymphocytes, suggests that in these groups at least, the homologous rosetting cells are thymus derived (Yu, 1975; Sheldon and Holborow, 1975a; Sheldon and Holborow, 1975b).

Further supportive evidence for the T cell nature of the homologous rosetting cell is given by in vitro results with lymphocyte cultures stimulated with phytohaemagglutinin, a T cell mitogen (Greaves et al., 1974b). Thus increased numbers of homologous and autologous rosetting cells have been reported in such cultures (Blecher, 1974; Sheldon and Holborow, 1975a).

This laboratory reported that increased numbers of homologous rosettes were present in the blood of patients with auto-immune haemolytic anaemia (Dewar et al., 1974).

In man therefore, the evidence indicates that homologous and autologous erythrocytic interaction with lymphocytes can be detected in normal subjects under defined conditions. The evidence suggests that these lymphocytes are probably T-lymphocytes.

6. Aims of the present investigation

It was hoped to confirm and extend our report of increased homologous rosetting cells in patients with clinically active A.I.H.A.

One objective was to investigate the nature of the central rosetting cell. In this chapter are reported investigations designed to identify the cell as T or B lymphocyte in terms of surface membrane markers. The results in A.I.H.A. are compared with similar investigations in patients with glandular fever. Parallel studies of the homologous rosettes produced by P.H.A. stimulation are also recorded.

A second objective was to find whether there was a correlation between the numbers of peripheral blood homologous R.F.C. and the clinical status of the patient. If a correlation was demonstrated then we wished to know whether treatment, either steroid therapy or splenectomy had any modifying influence.

The final objective was to define the physical conditions necessary for rosette formation and to determine the contribution of antigen sensitive cells, using Rh-null erythrocytes.

Precise identification of the rosetting cell in these terms is of importance. It enables discussion/

discussion of their role in the immune disturbance underlying A.I.H.A., in the context of recently reported experiments in auto-immune disorders.

RESULTS

1. Controls
2. Clinical correlation with peripheral blood levels of homologous and autologous R.F.C..
 - a) Group I
 - b) Group II
 - c) Group III
 - d) Group IV
 - e) Group V
 - f) Conclusions
3. Further observations in patients with A.I.H.A..
 - a) Patients in remission
 - b) Determination of surface markers in spleen and lymph nodes of 6 patients with A.I.H.A.
4. Observations on homologous and autologous rosettes in patients with glandular fever.
5. Homologous and autologous R.F.C. in other disorders.
6. Rosetting by phytohaemagglutinin stimulated lymphocytes.
- 7./

7. Identification of the rosetting cell
 - a) As a B-lymphocyte
 - b) As a T-lymphocyte
 - c) Light microscopic features.

8. Investigation of physical factors affecting homologous and autologous R.F.C. formation.
 - a) Red cell age
 - b) Effect of bovine serum albumin
 - c) Effect of human serum
 - d) The erythrocyte / white cell ratio
 - e) Effect of temperature
 - f) Effect of Rh-null erythrocytes
 - g) Homologous and autologous serum
 - h) Effect of abnormal red cells

RESULTS

1. Controls

The average, range and standard deviation for autologous and homologous rosettes in each control group is given in Table I.

GROUP	TYPE OF ROSETTE	AVERAGE	RANGE	STANDARD DEVIATION
YOUNG	HOMOLOGOUS	1.84	0.31 - 4.59	1.50
NORMALS	AUTOLOGOUS	1.00	0.08 - 2.30	0.75
ELDERLY	HOMOLOGOUS	1.56	0.16 - 3.75	1.1
NORMALS	AUTOLOGOUS	2.57	0.16 - 6.50	1.96
HOSPITAL	HOMOLOGOUS	1.48	0.13 - 4.64	1.17
CONTROLS	AUTOLOGOUS	1.63	0.26 - 7.96	3.16

Table I Levels of homologous and autologous rosettes in three control groups. Young normals - 11, Elderly normals - 18, Hospital controls - 10. (Figures - number in each group).

There is a wider range in the hospital and elderly controls especially with regard to autologous rosettes. Based on these figures the upper limit of normal for both types of rosette has been taken as 5% of the mononuclear suspension. Blood group differences did not influence the level of homologous rosettes, and none of the controls had positive antiglobulin tests.

2./

2. Clinical correlation with peripheral blood levels of homologous and autologous R.F.C.

Five groups were recognised and these are now described.

a) Group I

There were four patients in this group and they were identified by a common pattern of elevated homologous and autologous R.F.C. before splenectomy. Following this operation the R.F.C. returned to normal levels, to be followed by a pronounced and sustained increase in both forms of rosette.

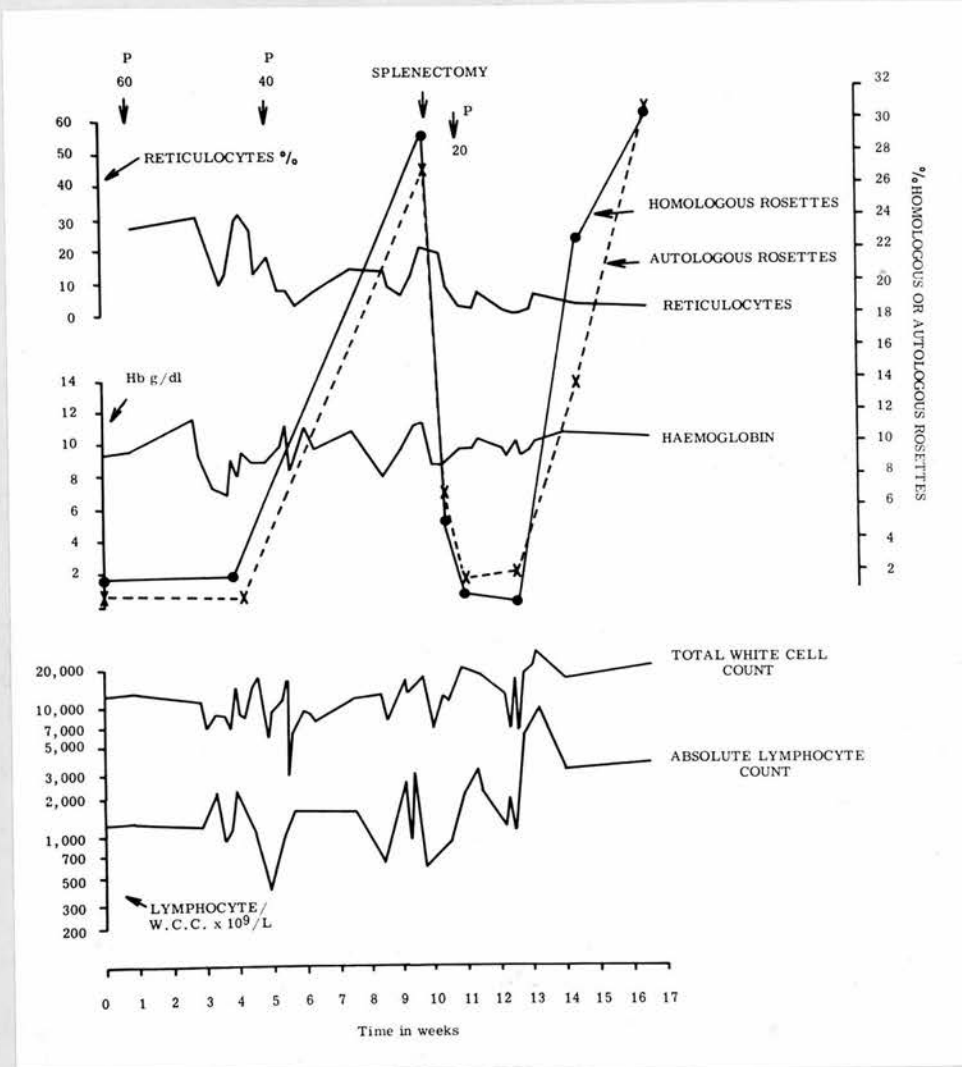
In addition to splenectomy, all four received steroids at some stage. Table II gives some pertinent details and Figs. I - IV illustrate the clinical course of the disease in each patient.

PATIENT	AGE	SEX	DIRECT ANTIGLOBULIN TEST TYPE	INDIRECT ANTIGLOBULIN TEST
E.McA.	64	F	IgG C	+
M.P.	73	F	IgG C	+
W.C.	14	M	IgG C	-
A.G.	71	F	C	-

Table II Some details of patients in Group I.

C = complement in all subsequent tables





DATE	ANTIGLOBULIN TEST		TYPE
	DIRECT	INDIRECT	
10. 6.75	1/320	+ (anti \bar{e})	C IgG
17. 9.75	1/320	+ (anti \bar{e})	C IgG
3.10.75	1/80	-	C

Figure I Group I Clinical Chart (E.McA.)

A high level of both autologous and homologous rosettes prior to splenectomy falls following operation. The sustained rise following this is illustrated.

In this and the subsequent fifteen figures

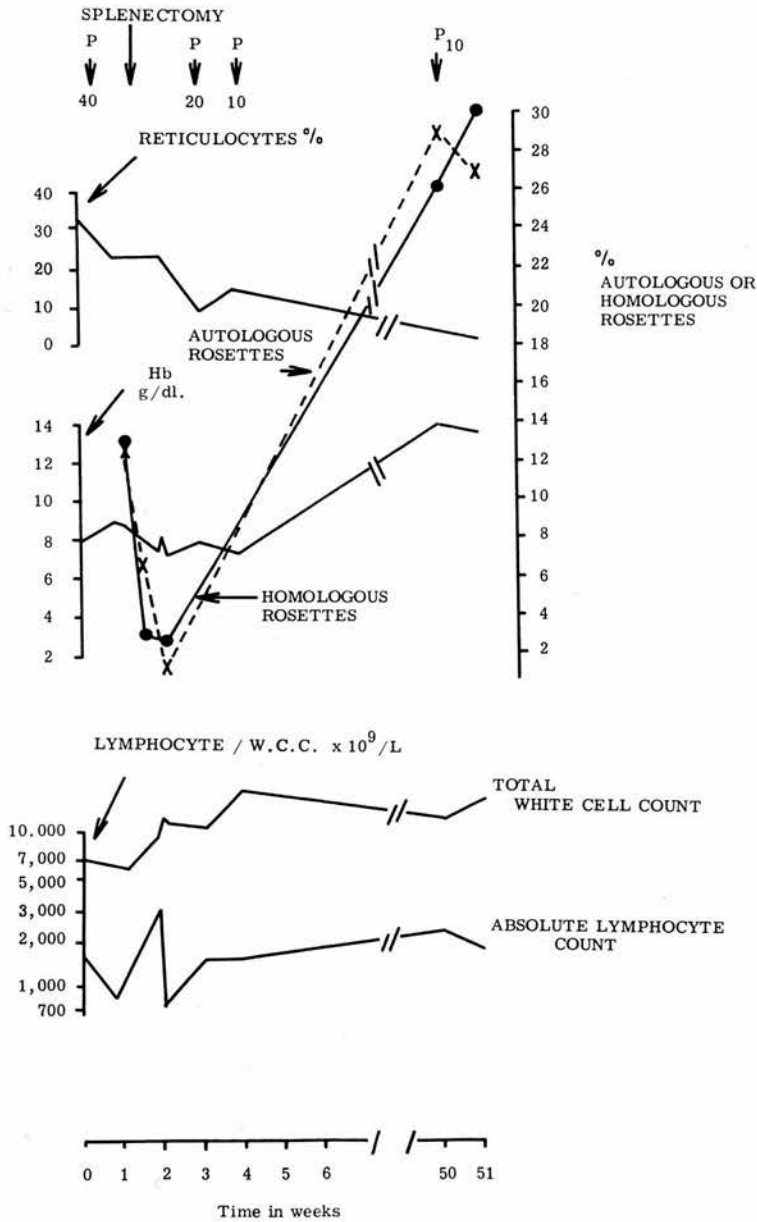
P = prednisolone; W.C.C. = white cell count;

Hb. g/dl = Haemoglobin/grams/decilitre;

C = the third component of complement;

- = negative; + = positive. The figures appertaining to prednisolone are dosages in milligrammes.

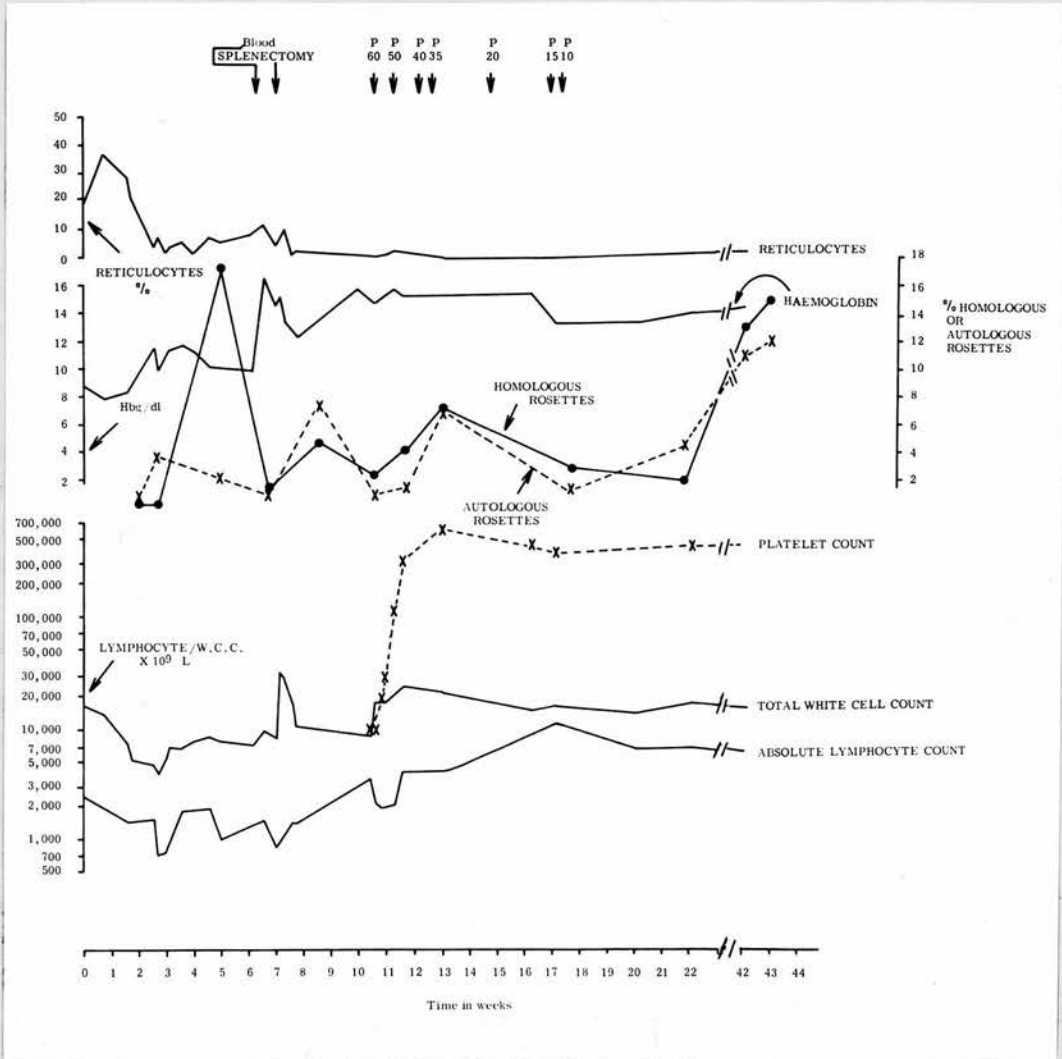
Within each group the legends give the initials of the patient studied. The first chart in each group is accompanied by a legend giving the general features. In subsequent charts in that group the legend indicates only differences from the first example. Attached to each chart are the antiglobulin tests performed over the period of observation.



DATE	ANTIGLOBULIN TEST		TYPE
	DIRECT	INDIRECT	
21.1.75	1/2000	+	IgG C
13.2.75	1/2560	+	IgG C
9.1.76	1/800	+	IgG C
16.2.76	1/300	+	IgG C

Figure II Group I Clinical Chart (M.P.)

The high levels of homologous and autologous rosettes are one year following splenectomy.



DATE	ANTIGLOBULIN TEST		TYPE
	DIRECT	INDIRECT	
23. 4. 74	1/80	-	IgG C
1. 7. 74	1/40	-	C
17.10. 74	-	-	

Figure III Group I Clinical Chart (W.C.)

This patient had an auto-immune thrombocytopenia soon after splenectomy (see text)

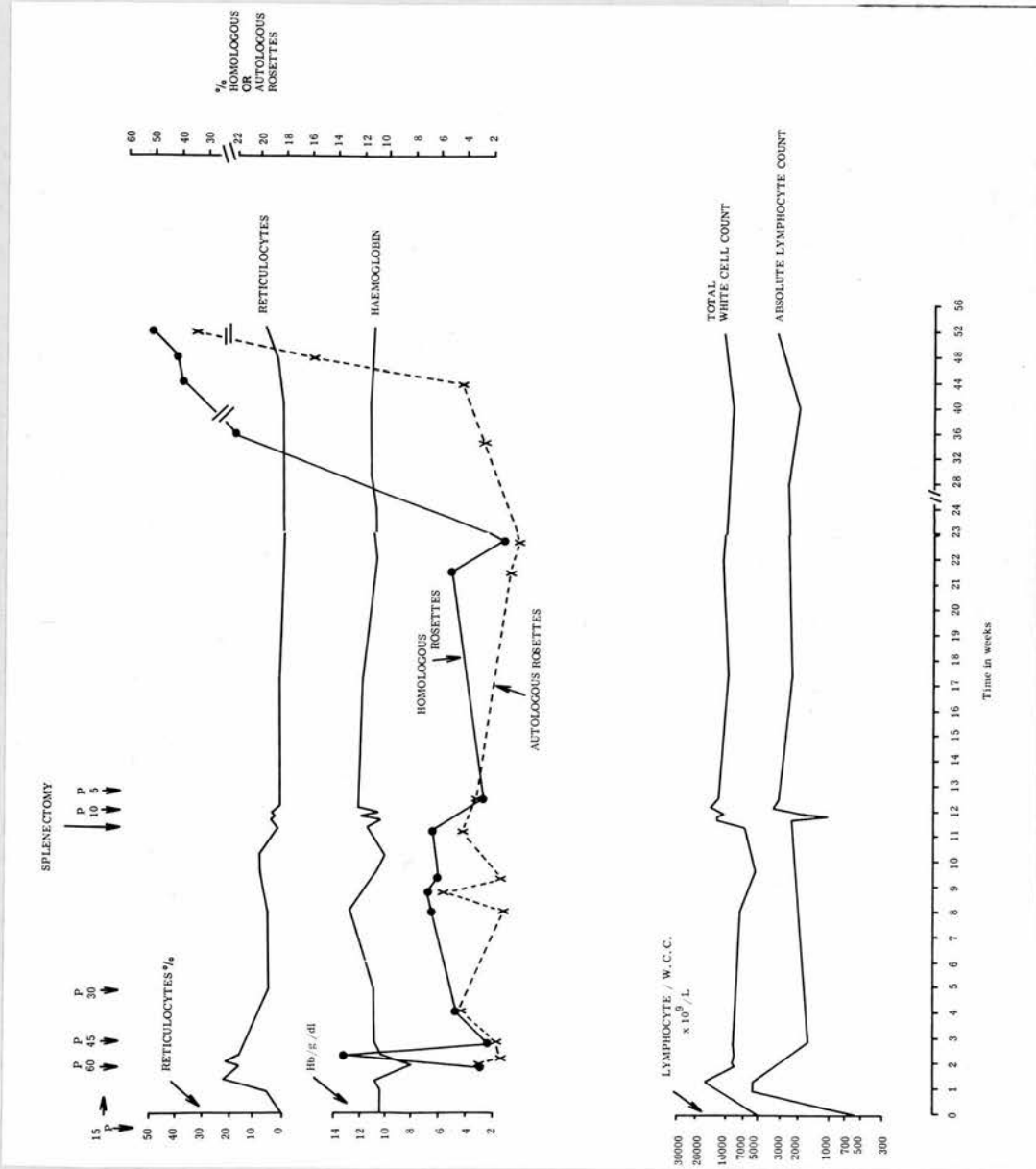


Figure IV Group I Clinical Chart (A.G.) The sustained rise in homologous rosettes occurred six months following splenectomy. Only this patient had a rising antigen globulin titre coincident with the increased rosettes.

In three patients, W.C., M.P., and E.McA., the rise in homologous and autologous R.F.C. occurred in the face of a falling antiglobulin titre. In A.G., there was a rise from 1/80 to 1/256, coincident with the increased level of rosettes.

In E.McA., M.P. and W.C. good correlation was noted between the levels of homologous and autologous R.F.C.. In A.G. differences between the two were recorded.

Lymphopenia was noted at various stages and occurred transiently in all patients following splenectomy.

i) Unusual Features

W.C. showed the pattern of elevated rosettes before splenectomy, falling to normal values and succeeded by a rise to abnormally high levels. During the period of 'rosette normality' he was readmitted with severe purpura. This was due to thrombocytopenia which responded to prednisolone. This episode of thrombocytopenia was not associated with any significant change in rosette forming cells.

b) Group II

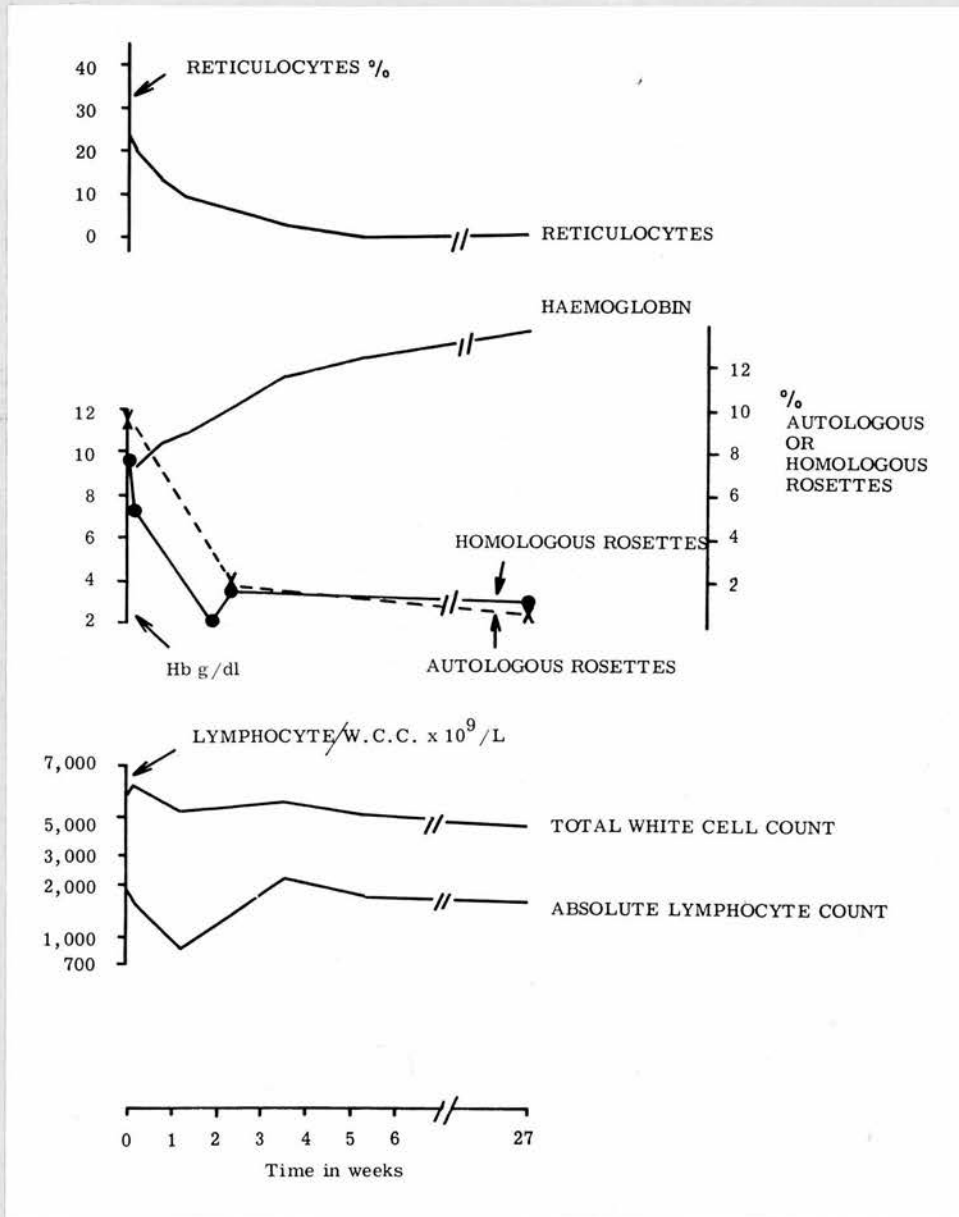
In all four patients a single acute haemolytic episode was associated with high homologous and autologous R.F.C. which fell to normal/

normal and remained normal as the disorder entered complete or partial remission. The only exception to this overall pattern was I.W. in whom a single isolated high autologous-R.F.C. was recorded twenty months following the initial illness.

The relevant details of the four patients are tabulated (III) and figures V - VIII show their clinical course.

PATIENT	AGE	SEX	DIRECT ANTIGLOBULIN TEST - TYPE	INDIRECT ANTIGLOBULIN TEST
I.H.	42	F	IgG C	-
E.H.	64	F	IgG	+
W.M.	83	M	IgG	+
I.W.	29	F	IgG C	-

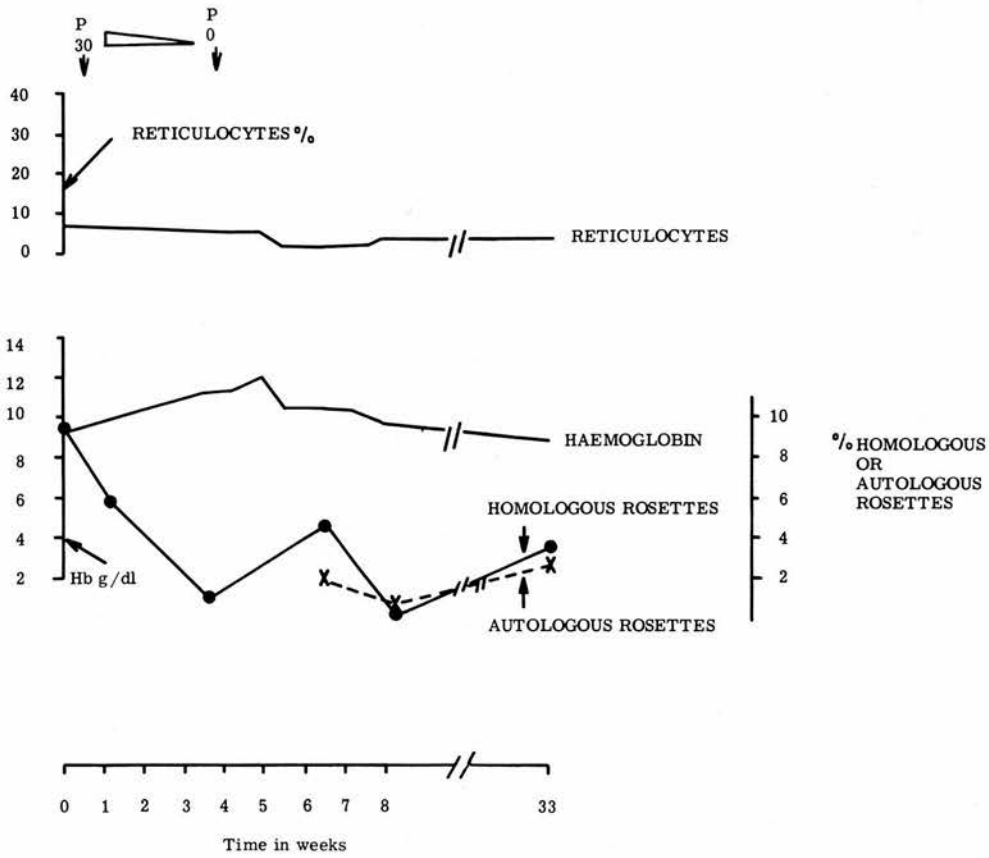
Table III Some details of patients in Group II.



DATE	ANTIGLOBULIN TEST		TYPE
	DIRECT	INDIRECT	
12. 5.75	1/40	-	IgG C
18.12.75	-	-	

Figure V Group II, Clinical Chart (I.H.)

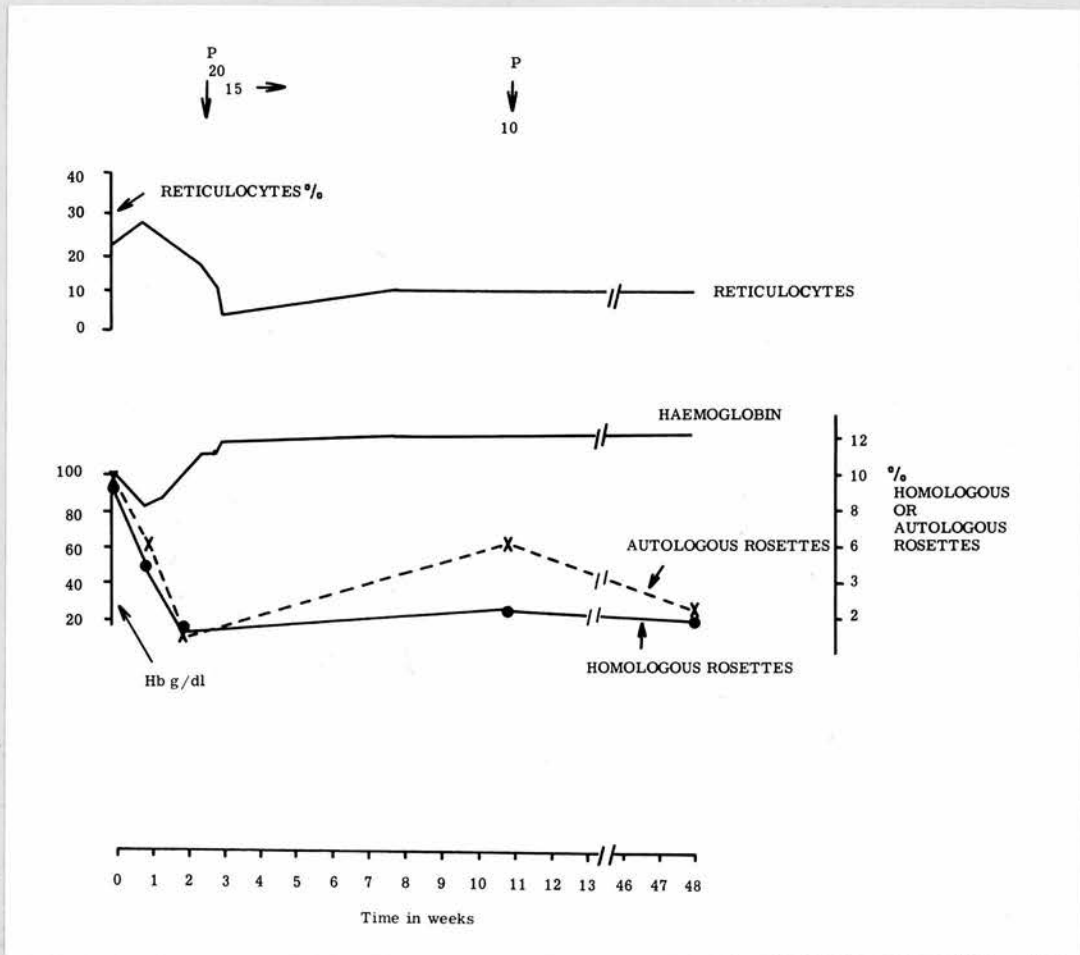
This figure shows the features of this group i.e. the single episode of high homologous and autologous rosettes with the initial acute illness.



DATE	ANTIGLOBULIN TEST		TYPE
	DIRECT	INDIRECT	
10.10.74	1/640	+	IgG
19.11.74	1/2560	+	IgG
4. 4.75	1/160	+	IgG

Figure VI Group II, Clinical Chart (E.H.)

This patient was initially studied during her second relapse.



DATE	ANTIGLOBULIN TEST		TYPE
	DIRECT	INDIRECT	
7. 2. 75	1/10240	+	IgG
6. 4. 75	1/10240	+	IgG
29.12.75	1/800	+	IgG

Figure VII Group II, Clinical Chart (W.M.)

This patient did not enter a complete remission.

DATE	ANTIGLOBULIN TEST		TYPE
	DIRECT	INDIRECT	
21. 6. 72	1/640	-	C
10. 1. 73	1/40	-	C
26. 11. 73	1/2560	-	IgG C
8. 5. 74	1/2560	-	IgG C
8. 10. 74	1/2560	-	IgG C
4. 12. 74	1/280	-	IgG
17. 12. 75	1/1600	-	IgG C

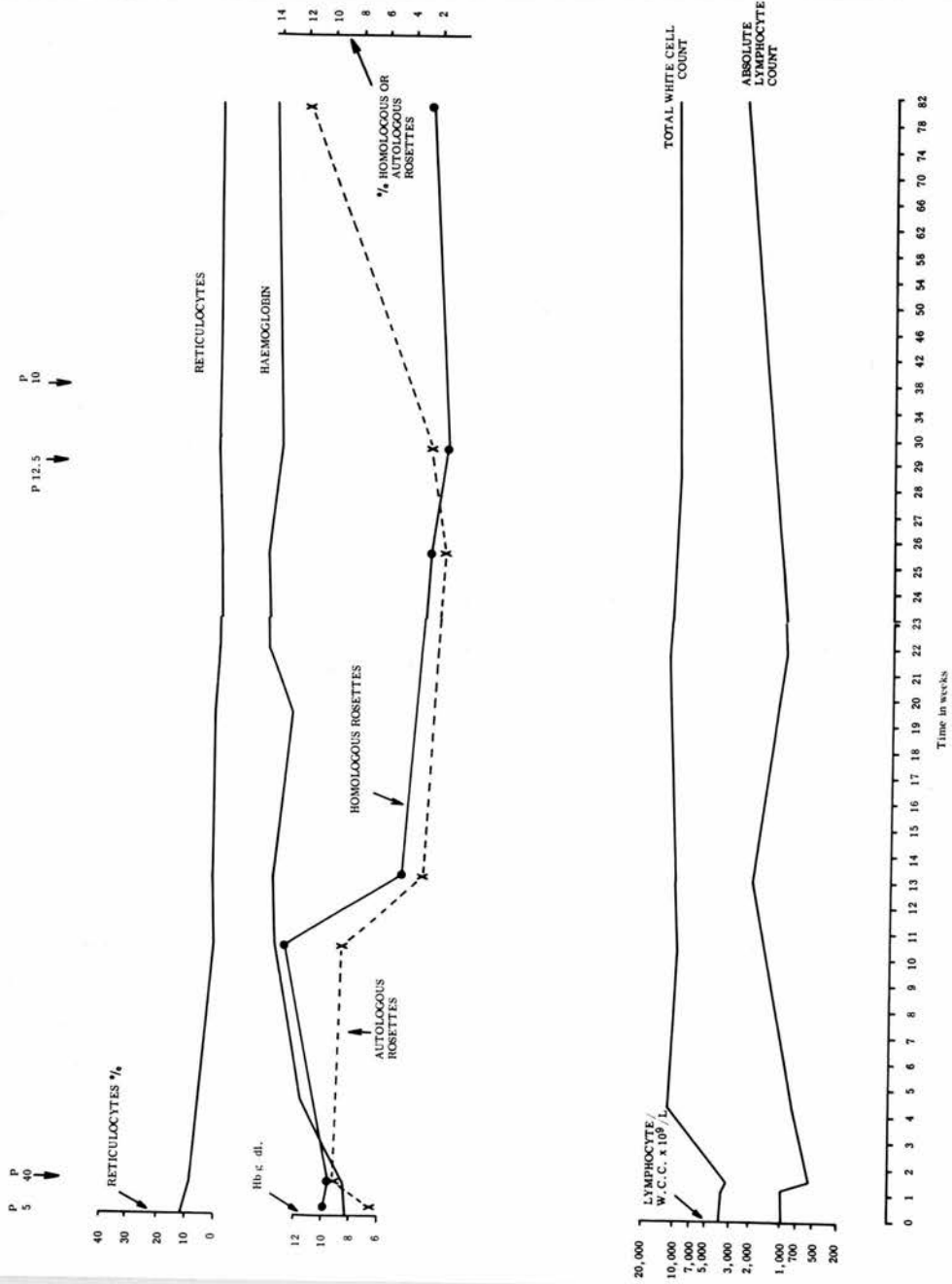


Figure VIII Group II, Clinical Chart (I.W.) In this patient the titre of the antiglobulin test did not fall.

E.H. was first studied during a second relapse. I.H. was not given steroids. The antiglobulin titres fell concurrently in three of the patients, the exception being I.W. There was good correlation between autologous and homologous R.F.C. in all patients apart from the single isolated final occasion in I.W.

i) Unusual Features

Patient I.H. showed very low values for peripheral blood T cells associated with elevation of homologous rosetting cells. This was noted at an early stage in her illness. The blood T cells rose steadily and the homologous rosetting cells fell as her clinical condition improved (Fig. IX). Consecutive tests for T and B cells have not been performed in all patients. This pattern has been noted in one other patient with A.I.H.A. and will be discussed later.

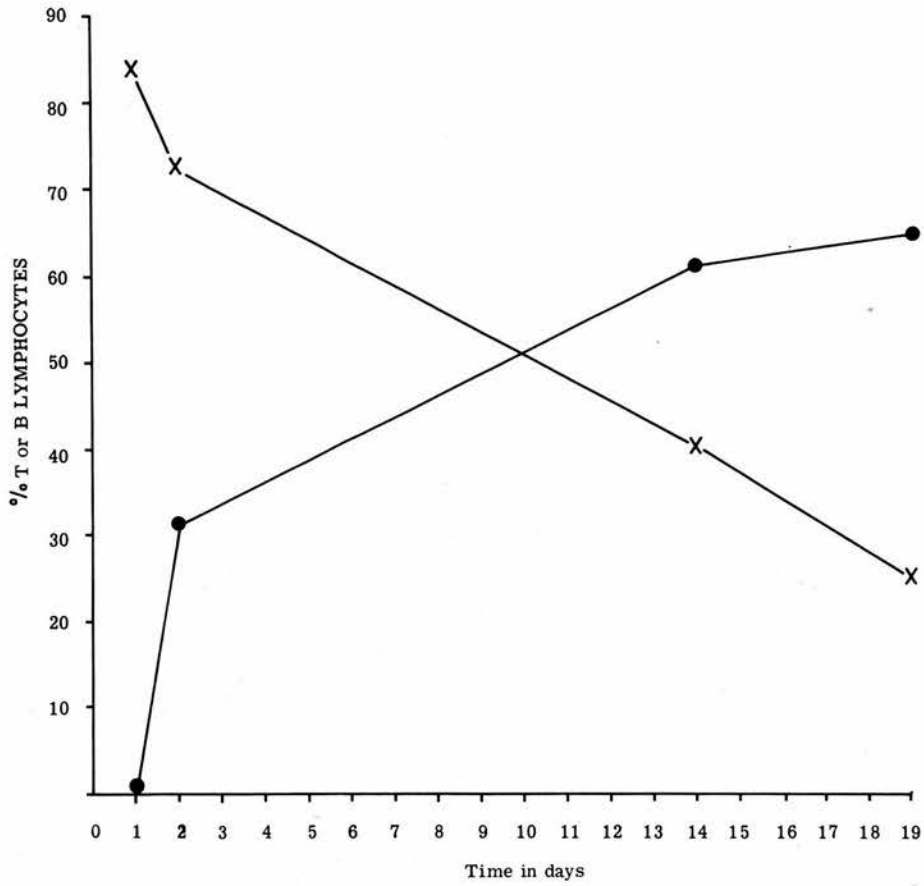


Figure IX Group II Unusual Features

During the acute phase of the disease a reversal of the normal T/B lymphocyte ratio was observed (● - T lymphocyte; X - B lymphocyte).

c) Group III

Three patients make up this group (B.S., M.B., E.McF.). A feature has been the intermittent rises in homologous and autologous R.F.C., at times reaching very high levels (Figs. X - XII). The patients have remained clinically well with little change in the peripheral blood, apart from a short lived episode in M.B. Table IV shows the pertinent details.

PATIENT	SEX	AGE	DIRECT ANTIGLOBULIN TEST - TYPE	INDIRECT ANTIGLOBULIN TEST
B.S.	F	84	IgG	-
M.B.	F	72	IgG	+
E. McF	F	74	IgG C	+

Table IV Some details of patients in Group III

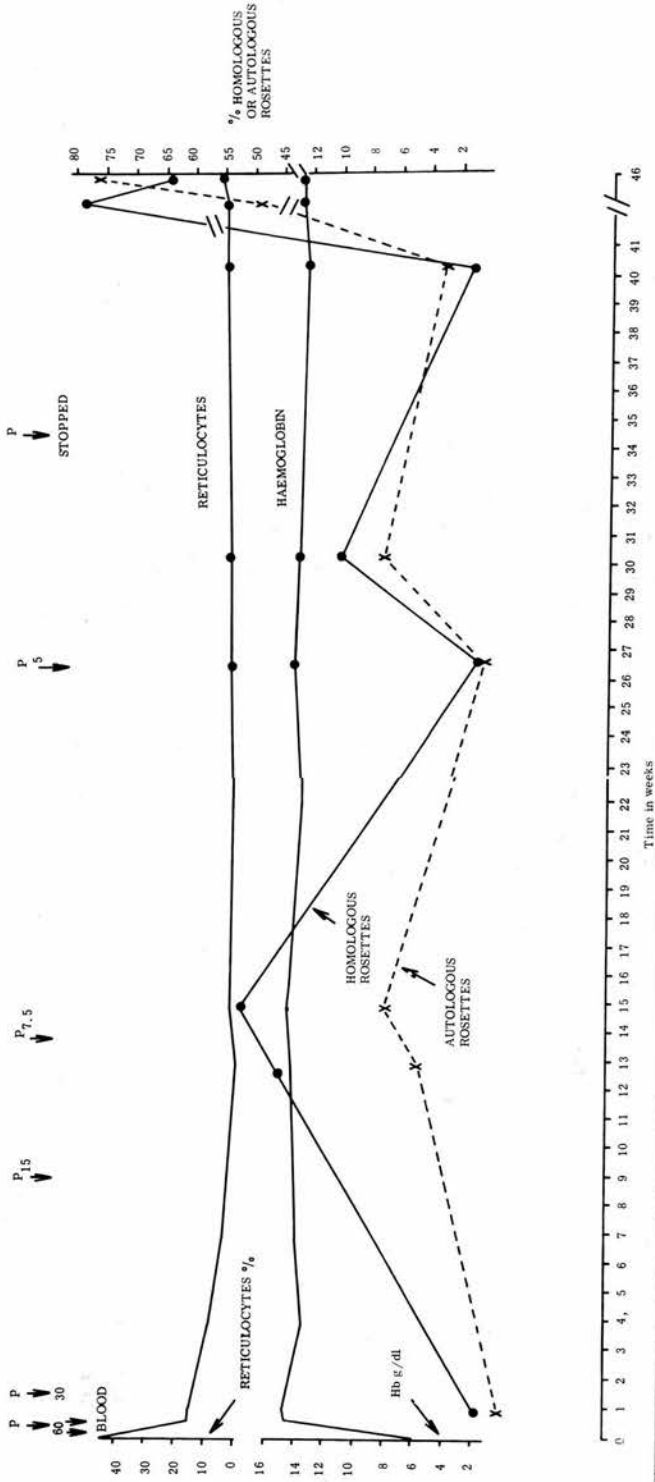
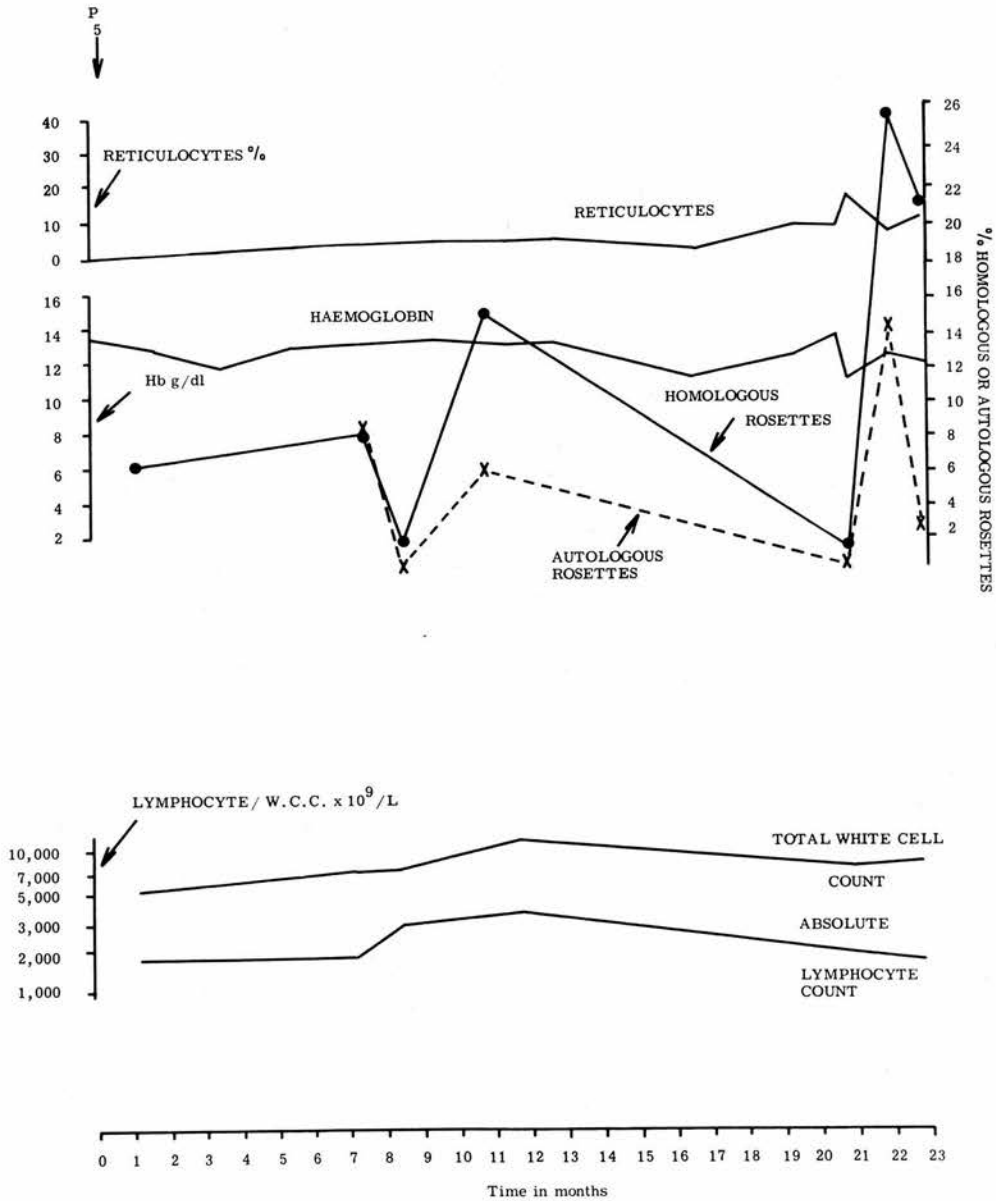


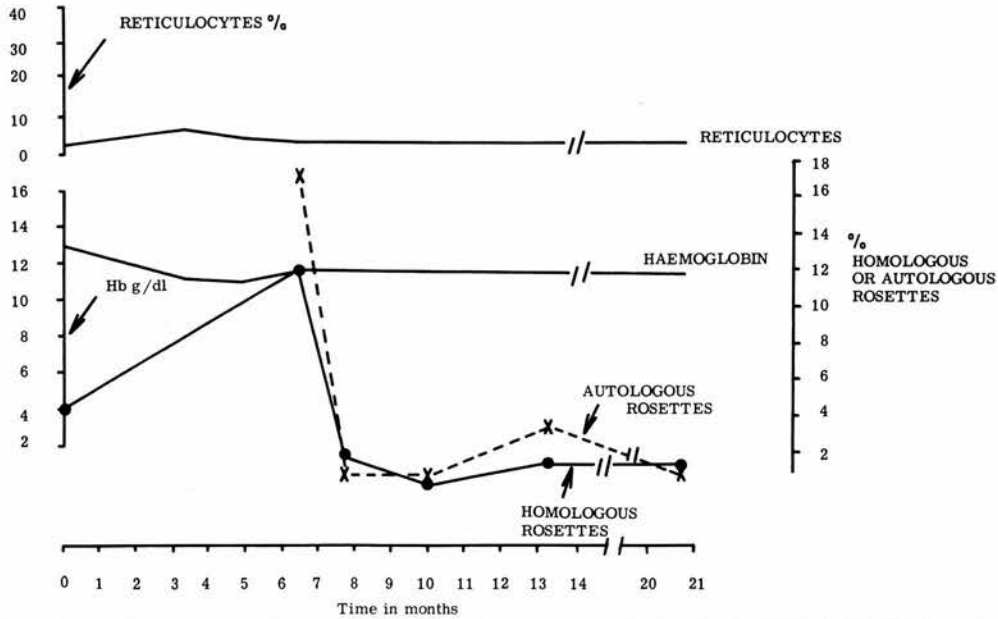
Figure X Group III, Clinical Chart (E.McF.) This chart shows the intermittent rises in autologous and homologous rosettes with no change in haemoglobin and a normal reticulocyte level.



DATE	ANTIGLOBULIN TEST		TYPE
	DIRECT	INDIRECT	
27.10.72	1/20,000	-	IgG
9. 3.73	1/2048	-	IgG
14.12.74	1/2560	-	IgG
16. 2.76	1/6400	+	IgG

Figure XI Group III, Clinical Chart (M.B.)

Lower levels of autologous rosettes noted in this patient particularly in the last measurements, when compared to homologous-R.F.C. levels.



DATE	ANTIGLOBULIN TEST		TYPE
	DIRECT	INDIRECT	
20. 3. 74	1/2560	-	IgG
4. 12. 74	1/2560	-	IgG
12. 3. 74	1/1600	-	IgG

Figure XII Group III, Clinical Chart (B.S.)

Only at one measurement were autologous and homologous rosettes raised in this patient.

In all patients the antiglobulin test has remained strongly positive. In M.B., coincident with the last recorded rise in both forms of rosette, the indirect antiglobulin test became positive for the first time.

As regards treatment none have undergone splenectomy and all for most of the period of study/

study were on little or no cortico-steroid.

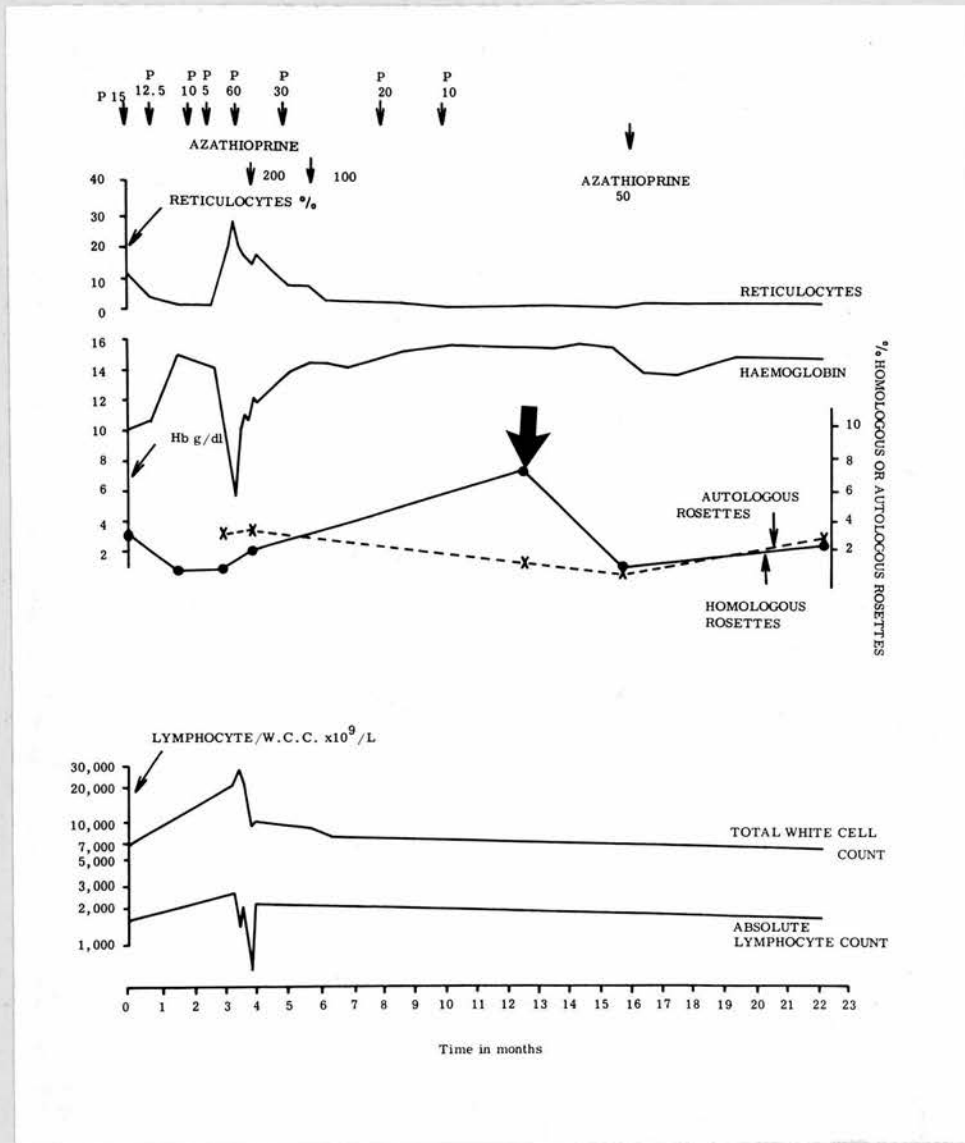
Though the autologous R.F.C. have mirrored the pattern of homologous R.F.C. they have tended to be lower.

d) Group IV

In these two patients there was little fluctuation in the homologous or autologous R.F.C. despite quite dramatic clinical and therapeutic changes (Table V, Figs. XIII, XIV).

PATIENT	AGE	SEX	DIRECT ANTIGLOBULIN TEST - TYPE	INDIRECT ANTIGLOBULIN TEST
A.W.	46	M	C	-
R.G.	83	M	IgG	+

Table V Some details of patients in Group IV.



DATE	ANTIGLOBULIN TEST		TYPE
	DIRECT	INDIRECT	
30.1.74	1/5120	+	IgG
14.2.74	1/1280	+	IgG
10.5.74	1/5120	+	IgG
21.2.75	1/160	+	IgG

Figure XIII Group IV, Clinical Chart (R.G.)

Despite two episodes of acute haemolysis, the homologous and autologous rosettes remained normal (apart from one point, arrowed).

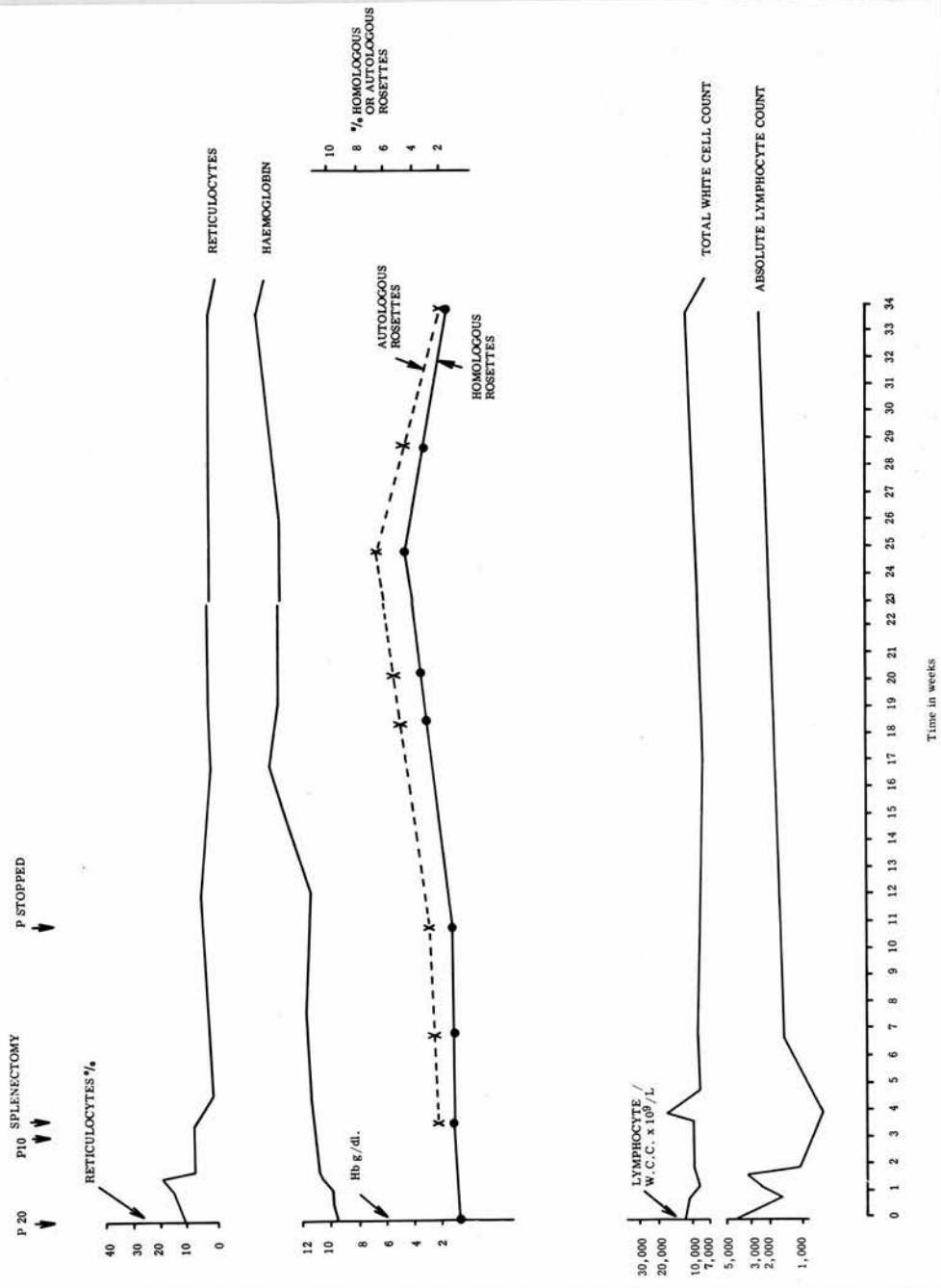


Figure XIV Group IV, Clinical Chart (A.W.) This patient had splenectomy, which was associated with lymphopenia.

At only two points of observation did the level of rosettes exceed the upper limits of normal established in the controls, homologous R.F.C. in the case of R.G. and autologous in the case of A.W.. This was despite severe presenting episodes of haemolysis in both patients, a relapse in the case of R.G. and a splenectomy in A.W.. Both patients received prednisolone and R.G. had azathioprine in addition. The antiglobulin test was different in both patients, being of low titre and due to complement in A.W. and high titre and due to IgG in R.G.. At the time of writing this test is negative in A.W. who is clinically well following splenectomy. R.G. has a positive antiglobulin test and is in clinical remission. In both patients there was a depression in the lymphocyte count when haemolysis was manifest.

Apart from one point (R.G.), levels of homologous and autologous R.F.C. were equivalent in both patients.

e) Group V

In T.K. (Table VI) there was correlation between the percentage of rosettes and the activity of the disorder, but it was not possible to fit him into the four groups described. He resembled Group I in that rosettes were high at presentation and/

and became lower after splenectomy. The rosettes remained low and did not show the dramatic rise noted after splenectomy in Group I patients. The patient remains well and his chart shows the clinical progress (Fig. XV). Later measurements, up to 18 months following splenectomy, not illustrated, have remained normal.

Lymphopenia was recorded and there was a marked temporary fall following splenectomy.

PATIENT	SEX	AGE	DIRECT ANTIGLOBULIN TEST - TYPE	INDIRECT ANTIGLOBULIN TEST
T.K.	M	12	IgG	+

Table VI Some details of patient in Group V.

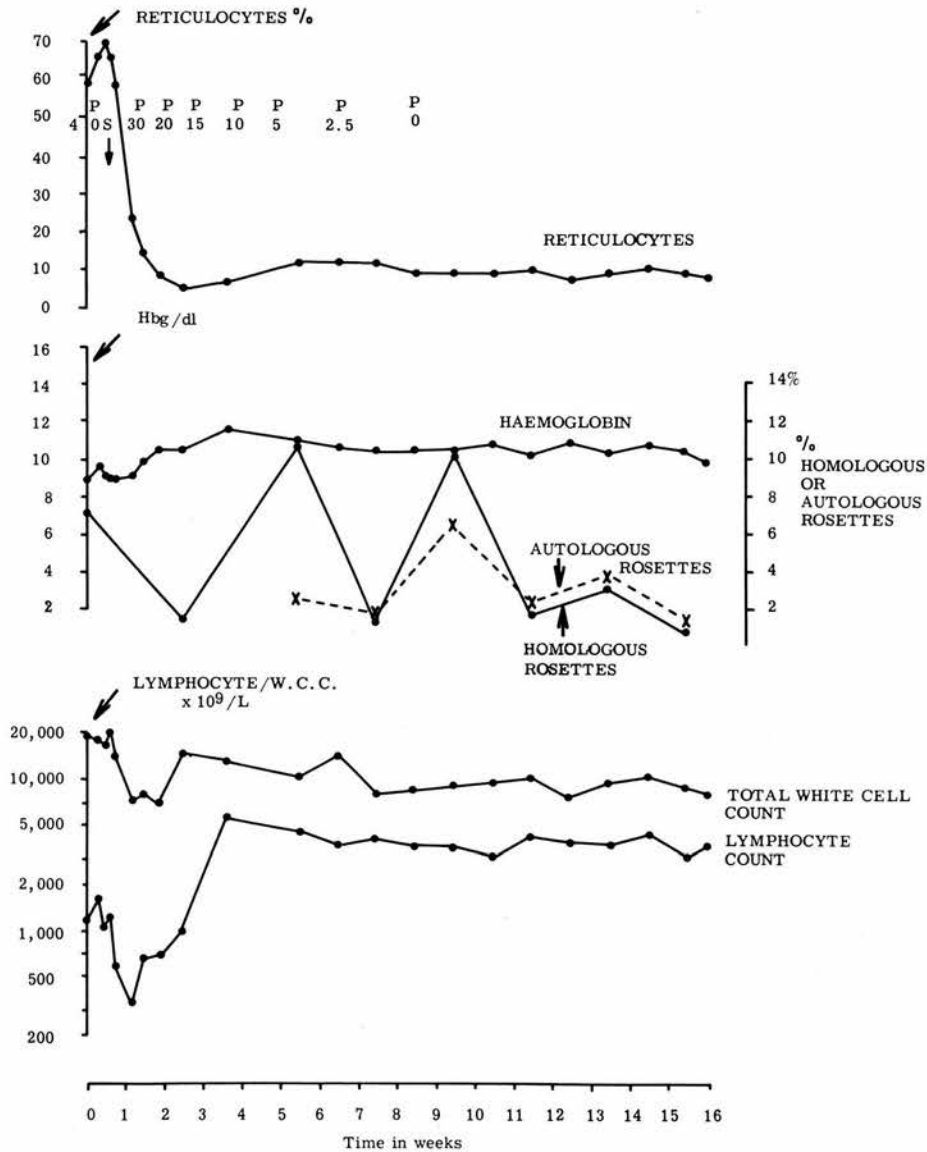


Figure XV Group V, Clinical Chart (T.K.)

This patient could not be fitted into the other four groups described.

i) Unusual features

T.K. was the other patient with A.I.H.A., in whom T and B lymphocytes were measured sequentially. A similar pattern to that noted in I.H. was shown by T.K. (Fig. XVI). In the latter patient this sequence of events occurred over a much longer time period than I.H. and could not be related to the levels of homologous or autologous rosetting cells.

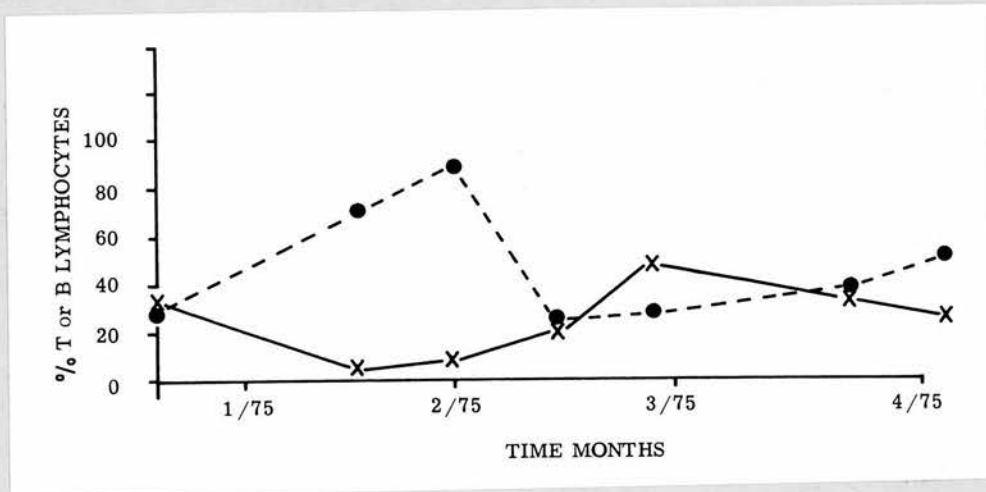


Figure XVI Group V Unusual features.

This patient showed a more prolonged period of reversal of the T/B lymphocyte ratio than the other patient showing a similar phenomenon.
(● - T lymphocytes, X - B lymphocytes).

f) Conclusions

In nine of the fourteen patients studied (two thirds of the total), a correlation was demonstrated between the rosette level in the peripheral blood and fluctuations in the disease process, either spontaneous or therapeutically induced.

3. Further observations in patients with A.I.H.A.

a) Patients in remission

In Table VII are shown random measurements of homologous and autologous R.F.C. in 11 patients with A.I.H.A. in remission. Remission was defined by clinical well being and normal peripheral blood measurements. In three patients (R.C., P.R., E.R.), the antiglobulin test was negative at the time of investigation. In only two patients (B.M. autologous M.F. homologous) was there an elevation of R.F.C.. On subsequent occasions in these two patients the values were normal.

PATIENT	HOMOLOGOUS ROSETTES	AUTOLOGOUS ROSETTES
A.B. 1	0.4	0.2
2	1.8	0.8
A.D.	1.0	1.3
R.C.	2.4	2.2
P.R.	0.4	0.6
F.K. 1	1.0	1.0
2	1.6	1.2
B.M. 1	1.5	8.8
2	1.8	0.7
3	1.0	1.0
M.F. 1	6.9	1.6
2	4.8	3.0
3	1.3	1.0
E.R.	2.2	1.2
J.W.	5.2	1.2
M.C.	2.6	4.6
J.D.	1.0	1.0

Table VII Homologous and autologous rosette levels in patients with quiescent auto-immune haemolytic anaemia.

b) Determination of surface markers in spleen and lymph nodes of 6 patients with A.I.H.A.

Though control values in these organs are not available it is apparent from Table VIII that in only one patient, (spleen, E.McA.), were large numbers of homologous and autologous R.F.C. found. In only two other instances (M.P. spleen and/

and lymph node) did the values exceed those found in the peripheral blood. Other mononuclear populations measured in some cases are included in the table.

AGE PATIENT	TISSUE EXAMINED	CONCURRENT THERAPY	HOMOLOGOUS ROSETTES %	AUTOLOGOUS ROSETTES %	T-LYMPHOCYTES	MONOCYTES	B-LYMPHOCYTES
72 M.P. (F)	Lymph Node	Steroid	1.3	5.8	51.2	22	21.4
72 M.P. (F)	Spleen	Steroid	2.9	6.9	56.2	19	34.2
68 E.McA (F)	Spleen	Steroid	25.2	36.5	49.0	5.5	9.5
73 W.F. (M)	Spleen	Nil	1	1	42	1	6
72 A.G. (F)	Spleen	Steroid	1.8	0.9	24.8	2.2	24.7
16 W.C. (M)	Spleen	Nil	1.1	0.8	N.D.	N.D.	N.D.
16 W.C. (M)	Lymph Node	Nil	3.9	1.3	N.D.	N.D.	N.D.
45 A.W. (M)	Spleen	Steroid	1	1	N.D.	N.D.	N.D.

Table VIII Cell surface markers in tissues derived from patients with A.I.H.A.. Peripheral blood measurements were not carried out on W.F. who had remarkably low monocytes and B lymphocytes in the spleen cell suspension.

(N.D. - not done; F - female; M - male).

4. Observations on homologous and autologous rosettes in patients with glandular fever

In Table IX are shown the levels of homologous and autologous R.F.C. in eight patients with glandular fever. All cases showed elevations of both types of rosette above control levels. None had positive antiglobulin tests at the time of investigation.

PATIENT	% HOMOLOGOUS ROSETTES	% AUTOLOGOUS ROSETTES
R.S.	16.0	18.0
L.S.	5.3	9.9
S.C.	33.5	26.0
McR	19.0	15.0
A.L.	10.2	13.3
R.C.	15.5	11.0
L.F.	13.0	22.5
R.W.	53.5	10.0

Table IX Homologous and autologous rosette levels in the peripheral blood of patients with glandular fever.

In three patients sequential measurements were carried out. Two were given steroid and the administration of this drug was associated with a progressive diminution in the number of rosetting cells./

cells. This type of response is illustrated in Fig. XVII.

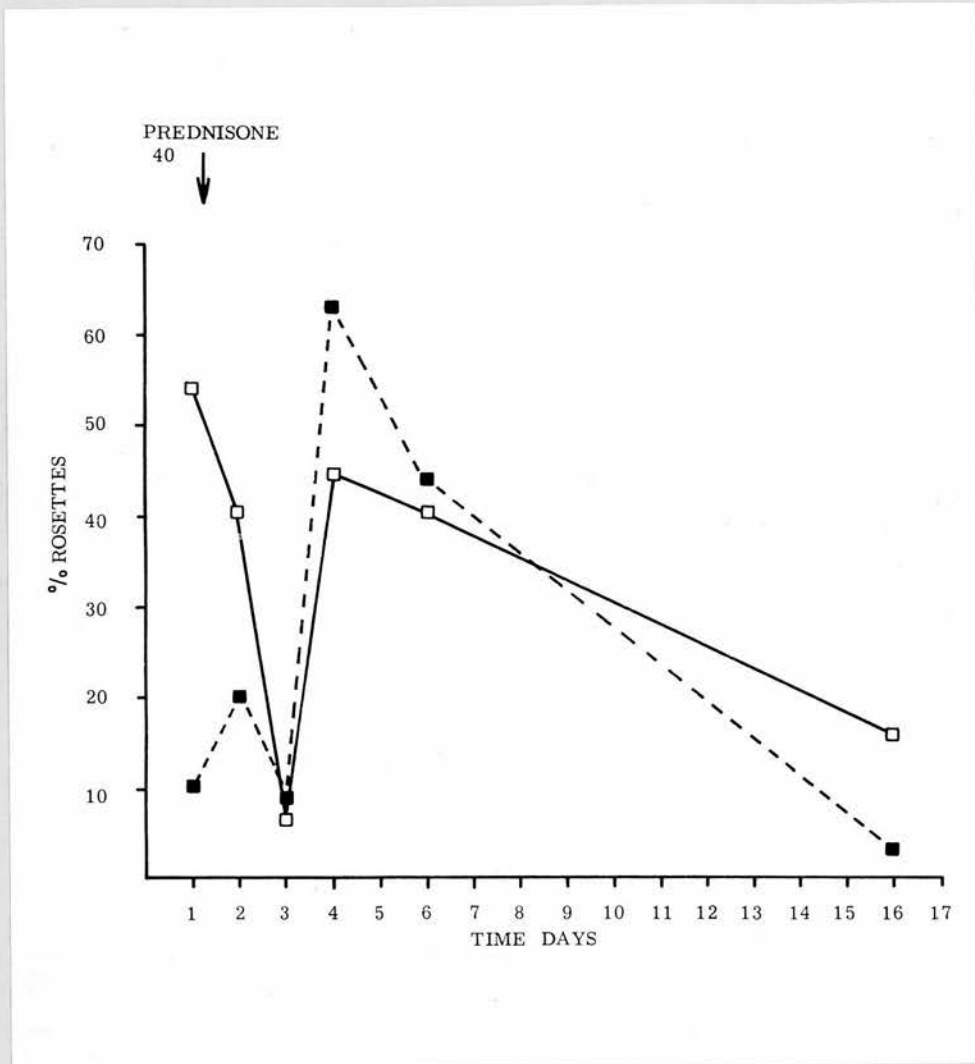


Figure XVII Autologous and homologous rosettes in a patient with glandular fever

Sequential measurements are shown. Of particular note is the marked drop of homologous rosettes following prednisone therapy.

(■ - autologous rosettes; □ - homologous rosettes)

In Table X are shown the corresponding measurements of/

of T and B lymphocytes and monocytes in seven of these eight patients.

PATIENT	B-LYMPHOCYTES %	T-LYMPHOCYTES %	MONOCYTES %
R.S.	29.0	80.0	5.0
L.S.	10.0	46.3	1.0
S.C.	11.0	74.0	1.0
McR.	18.0	37.5	N.D.
A.L.	22.5	52.0	N.D.
R.C.	12.0	53.0*	5.0
L.F.	17.0	77.5	8.0

* Results may be low due to excessive clumping of cells.

Table X T and B lymphocytes and monocyte levels in the peripheral blood in glandular fever. Values T lymphocytes 37.5 - 80% (mean 60%); B lymphocytes from 11 - 29% (mean 17%); monocytes 1 - 8% (mean 8%).

(Controls T \rightarrow $53.2 \pm 10\%$; B \rightarrow $23.2 \pm 8\%$; monocytes \rightarrow $15 \pm 5\%$). ND - not done.

There was a significant elevation of T lymphocytes in only three cases. Monocytes, in the five cases measured, were all low.

5. Homologous and Autologous R.F.C. in other disorders.

White cell disorders (Table XI). Four different white cell diseases are given together with/

with the corresponding membrane markers. In none was there elevation of either homologous or autologous R.F.C.. The populations of cells studied in terms of T - B lymphocyte and monocyte is given in all cases.

PATIENT AGE	DISORDER	HOMOLOGOUS ROSETTES %	AUTOLOGOUS ROSETTES %	T LYMPHOCYTE %	MONOCYTE %	B LYMPHOCYTE %
68 (F)	PLASMA CELL LEUKEMIA	1.5	0.5	3	1	3
67 (M)	MONOCYTOSIS	0.5	0.5	3	39	13
8 (M)	ACUTE LYMPHOCYTIC LEUKEMIA	0.0	0.2	2.5	1.0	26.5
72 (M)	CHRONIC LYMPHATIC LEUKEMIA	0.7	0.3	4.4	N.D.	71.7
65 (F)	CHRONIC LYMPHATIC LEUKEMIA	5.0	1.5	23	7	75
46 (M)	CHRONIC LYMPHATIC LEUKEMIA	<1	0.5	19	<5	<5
54 (M)	MONOCYTOSIS	2	2.3	7	56	23
33 F ₁	PERSISTENT	2	1.8	84	<5	20
2	LYMPHOCYTOSIS	1	1.3	78	<5	19

Table XI Cell membrane markers in various disorders.

In none of these examples was there elevation of homologous or autologous rosetting cells (N.D. - not done; M - male; F - female).

6. Rosetting by Phytohaemagglutinin stimulated lymphocytes

When human mononuclear peripheral blood cells were incubated with P.H.A. for 18 hours there was an increase in the number of rosetting cells. Rosettes formed with homologous red cells were increased, as were those with sheep erythrocytes (Fig. XVIII and XIX). Three patients with A.I.H.A. and two controls were studied.

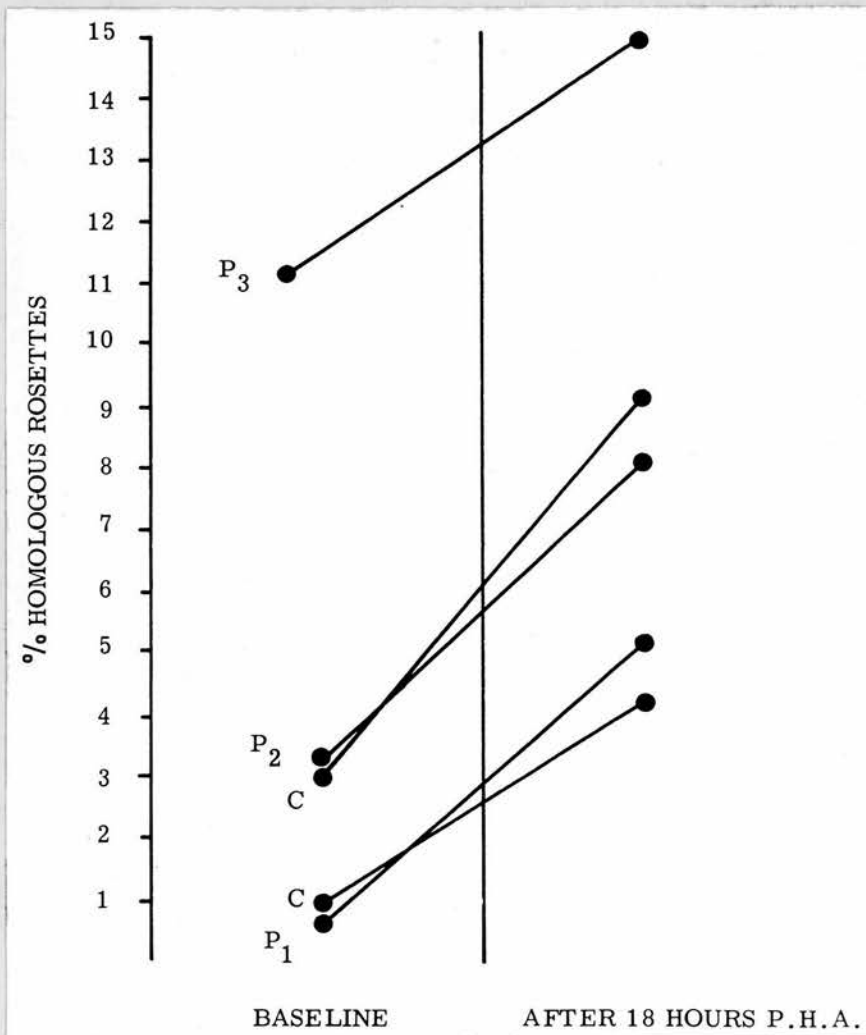


Figure XVIII The effect of phytohaemagglutinin on the levels of homologous rosettes with short term culture. (P = patient (3); C = control (2)).

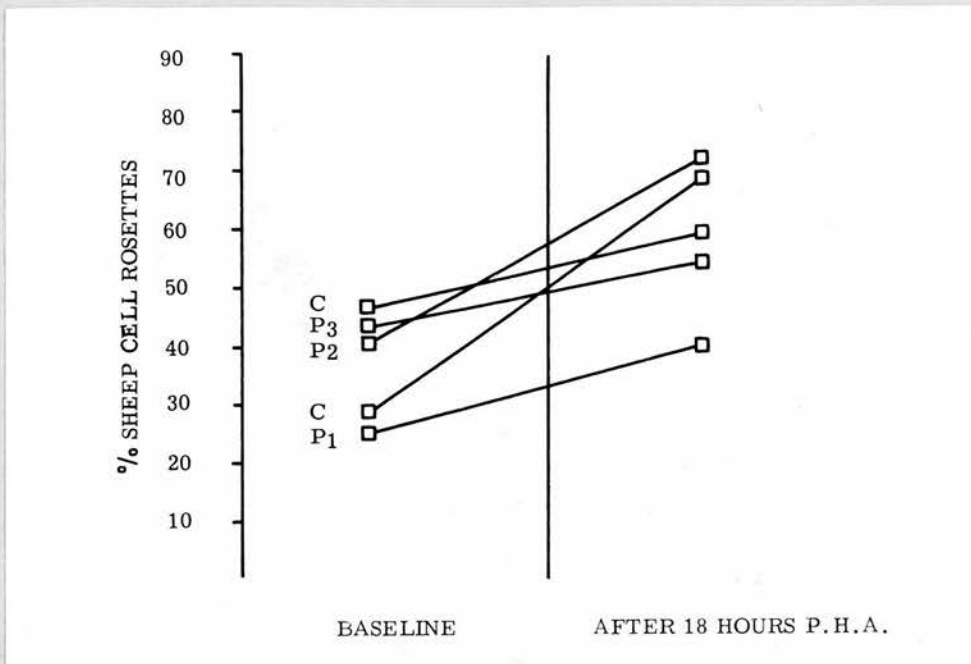


Figure XIX The effect of phytohaemagglutinin on the level of sheep cell rosettes with short term culture.

(P - patient (3); C - control (2)).

In four subjects the effect of phytohaemagglutinin was noted at 18, 24, 48 and 72 hours. All showed a similar pattern with a peak response occurring at 48h. During this period no significant difference in the proportion of T lymphocytes, as shown by the ability to form rosettes with sheep erythrocytes, was noted (Fig. XX). Cytochalasin B (5µg/ml) was added in two experiments. The result of adding this drug was inhibition of the expected rise in autologous and homologous R.F.C. These experiments were on controls only.

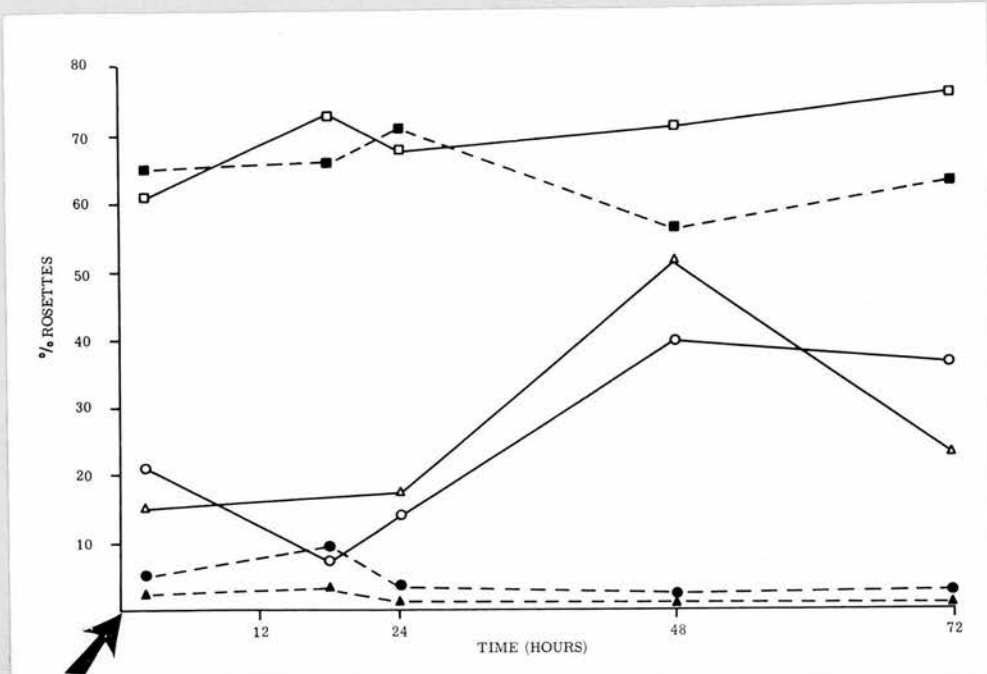


Figure XX The effect of phytohaemagglutinin on the levels of sheep cell, homologous and autologous rosettes with prolonged culture. Arrow indicates initiation of culture.

- - Sheep cell rosette (test)
- - Homologous rosette (test)
- △ - Autologous rosette (test)
- - Sheep cell rosette (control)
- - Homologous rosette (control)
- ▲ - Autologous rosette (control)

7. Identification of the rosetting cell

a) As a B lymphocyte

If the rosetting cells were B lymphocytes they would be expected to carry surface immunoglobulin. In Table XII are the results of combined fluorescence and/

and rosetting carried out on 74 homologous rosettes, identified in 800 cells from four patients with A.I.H.A.. No rosettes were fluorescent positive. An illustration (Fig. XXI) shows the capping fluorescence encountered in these preparations.

TOTAL NUMBER OF CELLS	FLUORESCENT POSITIVE CELLS	HOMOLOGOUS ROSETTES	FLUORESCENT POSITIVE ROSETTES
800	228	74	0

Table XII The incidence of homologous rosettes which carry surface immunoglobulin in patients with A.I.H.A.

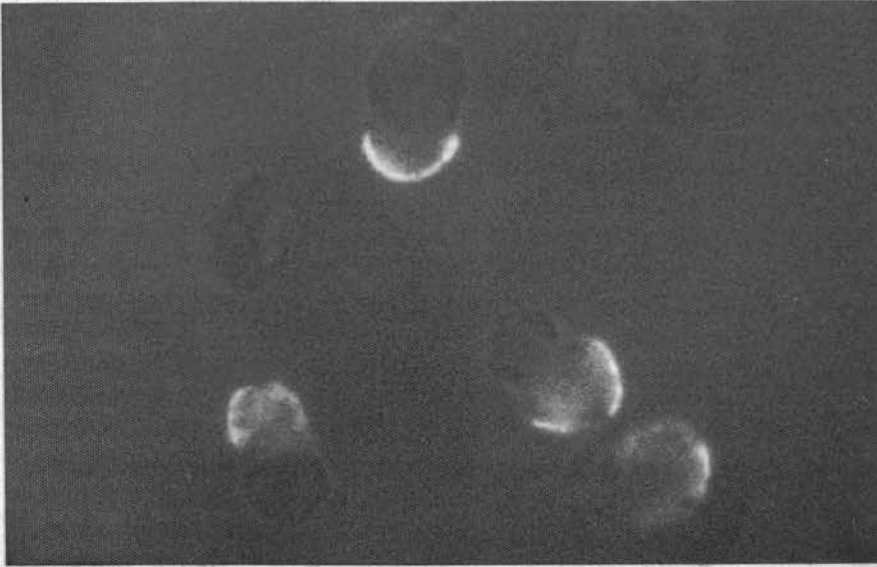


Figure XXI Lymphocytes demonstrated by fluorescence with ample surface immunoglobulin. The cell outline can also be made out.

In patients with glandular fever, rosetting cells were examined for the presence of surface immunoglobulin by fluorescence with fluorescein conjugated (F.I.T.C.) immunoglobulin and for the Fc receptor by the addition of aggregated IgG (F.I.T.C.). The results showed that the great majority of rosetting cells failed to react with either of these reagents (Table XIII).

TYPE OF PREPARATION	TOTAL NUMBER OF CELLS EXAMINED	FLUORESCENT POSITIVE CELLS	TOTAL NUMBER OF ROSETTES	FLUORESCENT POSITIVE ROSETTES
Homologous Rosettes + Surface Fluorescence	600	85	39	3
Sheep Cell Rosettes + Surface Fluorescence	300	22	185	2
Autologous Rosettes + Surface Fluorescence	100	22	11	0
Homologous Rosettes + Aggregated IgG Fluorescence	200	12	12	2

Table XIII The incidence of homologous rosettes in glandular fever which carry either surface immunoglobulin or express an Fc receptor.

b) As a T lymphocyte

In these experiments sheep erythrocytes were mixed with homologous or autologous red cells. The binding of sheep erythrocytes is a marker for the T lymphocyte in man. Since sheep erythrocytes can be distinguished from human red cells by their size, this technique is a useful method for detecting T lymphocytes.

In eight experiments with cells derived from five patients with A.I.H.A., most had elevated homologous rosettes. There was also a parallel elevation/

elevation in the mixed rosette, composed of both sheep and homologous red cells. It was surprising that the values for the mixed rosettes were higher than for homologous and the possible reasons for this are discussed later.

Virtually all the homologous R.F.C. took part in the mixed reaction and very low numbers of purely homologous rosettes were noted in the mixed suspension (Fig. XXII). This data supports the T lymphocyte origin for the rosetting cell.

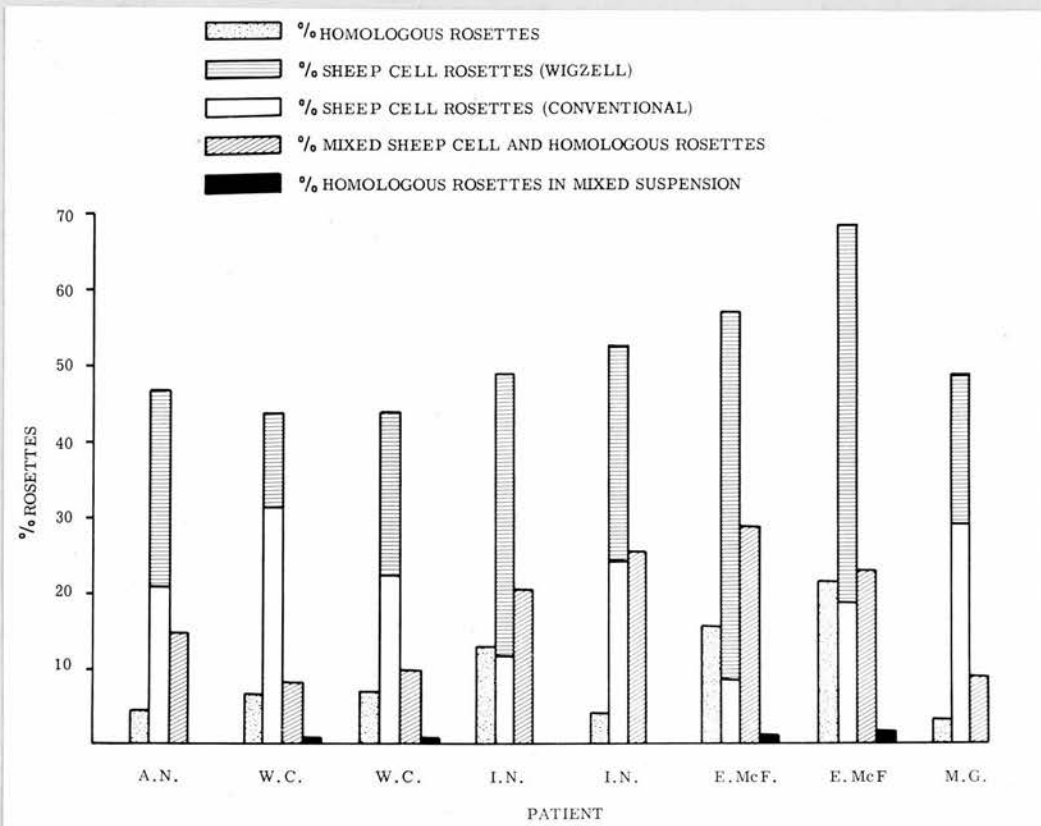


Figure XXII The results of measuring homologous rosettes alone and mixed sheep and homologous rosettes in five patients with A.I.H.A.. Virtually all the homologous rosettes in the mixed suspension were shown to carry sheep erythrocytes in addition.

Almost similar results were obtained in glandular fever (Figs XXIII, XXIV).

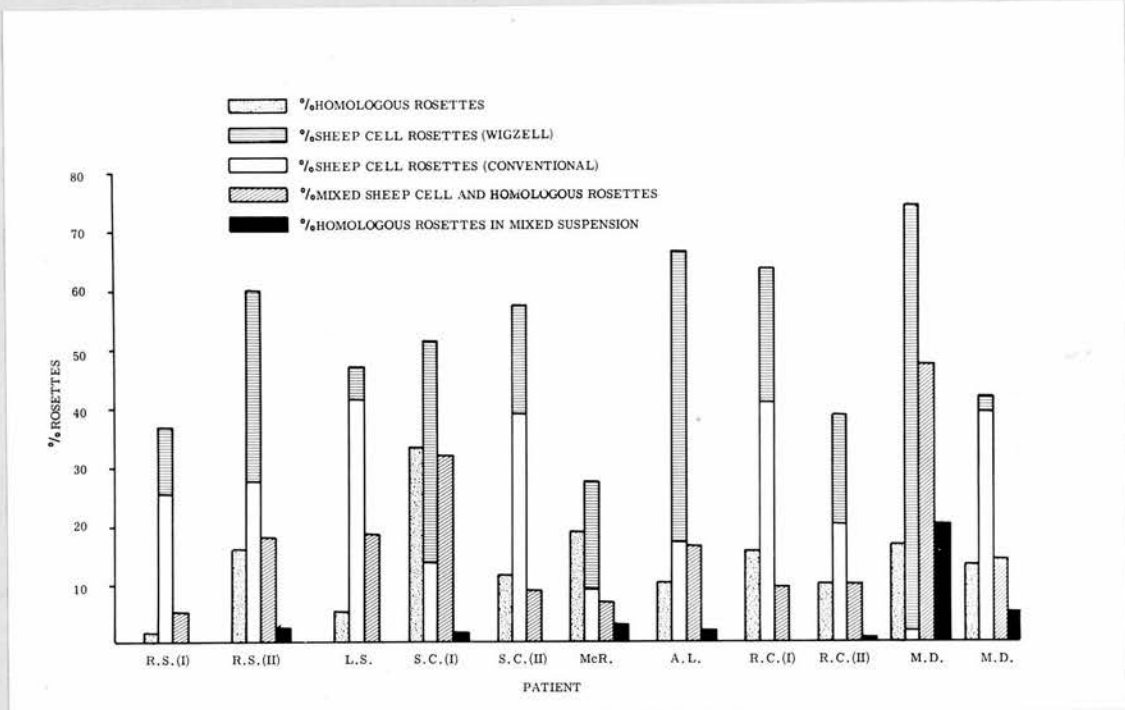


Figure XXIII The results of measuring homologous rosettes alone and mixed sheep and homologous rosettes in seven patients with glandular fever are shown.

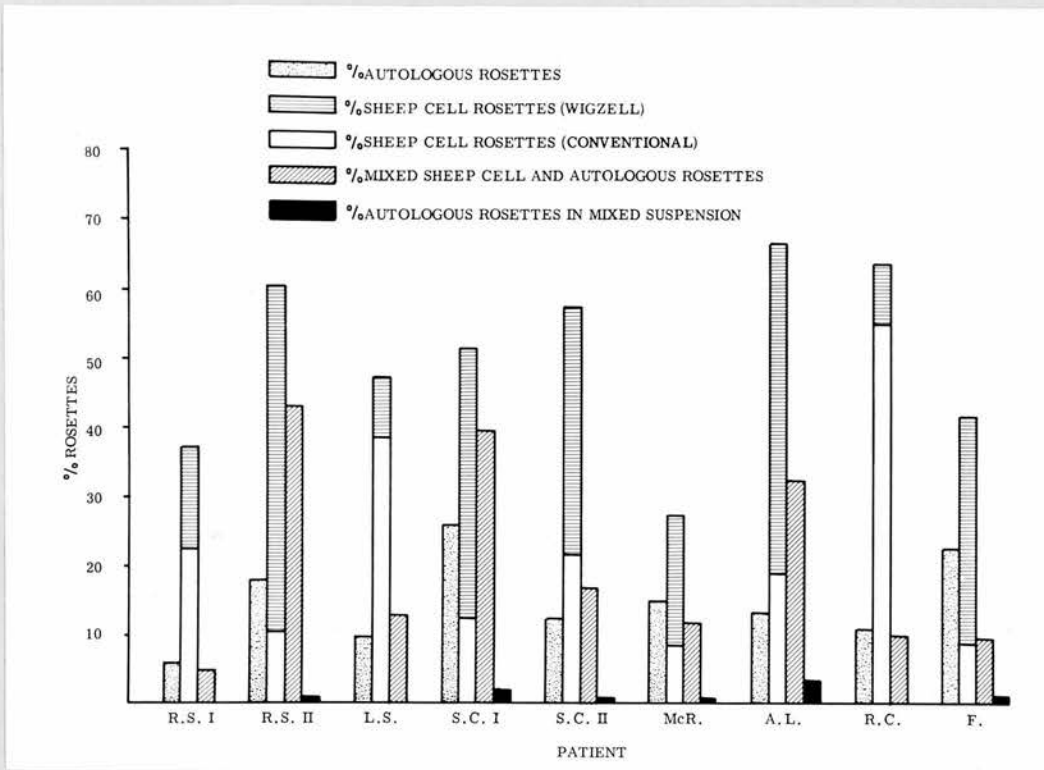


Figure XXIV The results of measuring autologous rosettes alone and mixed sheep and autologous rosettes in seven patients with glandular fever.

c) Light microscopic features of homologous - R.F.C. and sheep cell rosettes in A.I.H.A., glandular fever and P.H.A. stimulated cultures.

i) A.I.H.A. and Glandular Fever

In both these conditions similar features were noted. In phase contrast preparations homologous/

homologous and autologous R.F.C. usually had a maximum of five erythrocytes attached (Fig. XXV). Treatment of the preparations with glutaraldehyde preserved the rosettes which were then examined, no difference was noted in the number of red cells forming these rosettes.

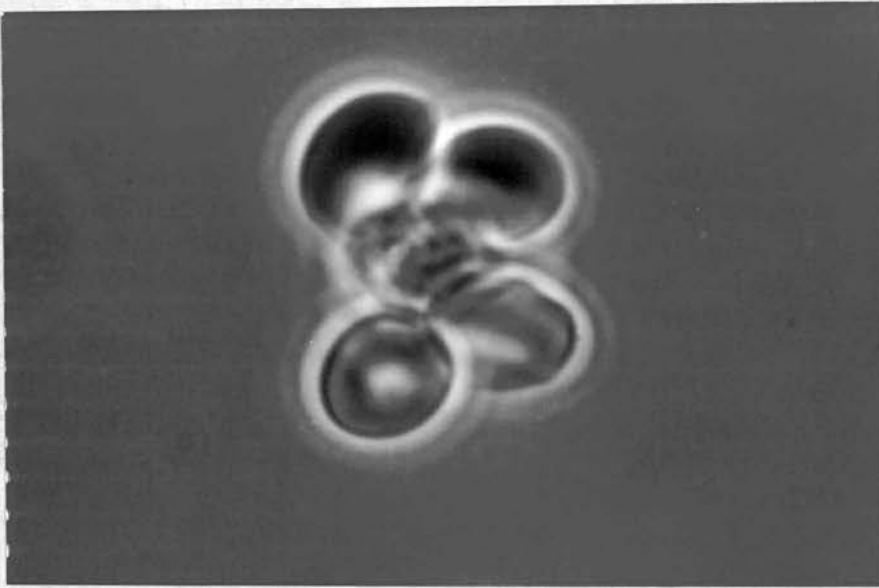


Figure XXV X 1,600 This shows a homologous rosette. Phase contrast preparation, cell derived from patient with A.I.H.A.

Sheep cell rosettes varied from those with 3 - 4 S.R.B.C., to complete morulae (Fig. XXVI). The attachment of red cells in these rosettes was more intimate than those in homologous/autologous preparations.

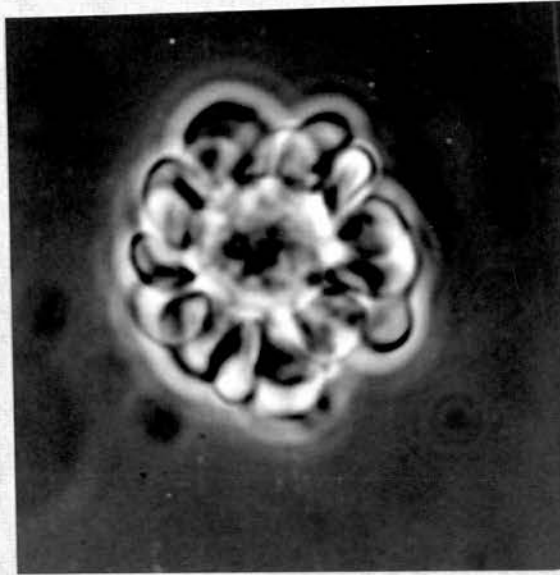


Figure XXVI X 1,600 This is a sheep cell rosette from a patient with A.I.H.A. (Phase contrast).

In mixed rosettes these features were maintained. Thus sheep cells were firmly attached, whereas the human red cells had a looser association (Fig. XXVII).

Usually only one, and at the most two (Fig. XXVIII) human red cells were attached.

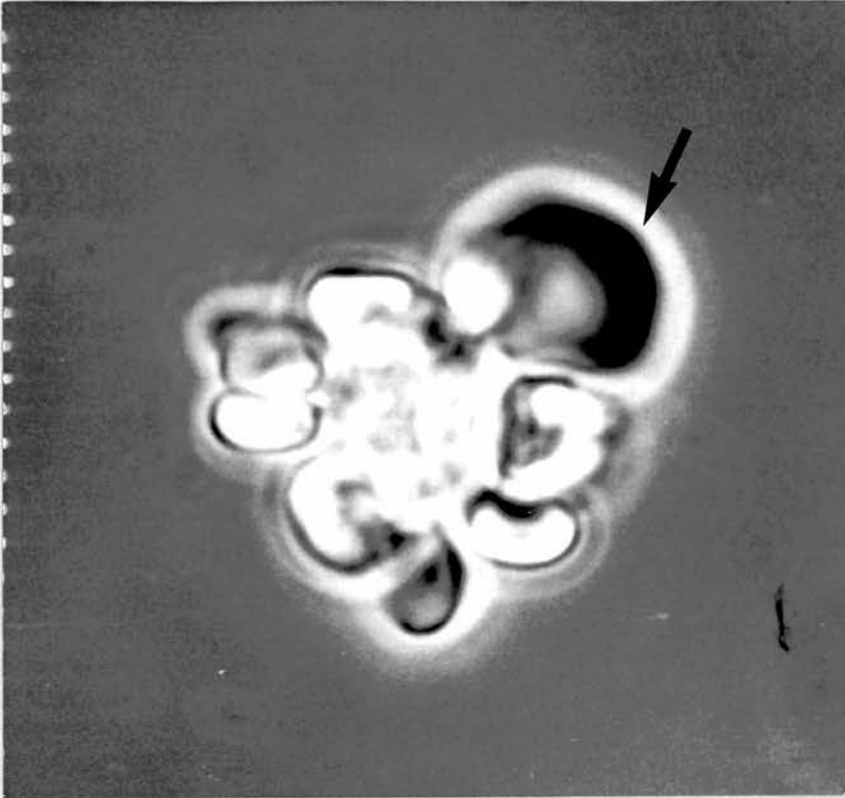


Figure XXVII X 2,000 This is a mixed sheep and homologous (arrowed) rosette from a patient with A.I.H.A. (Phase contrast).

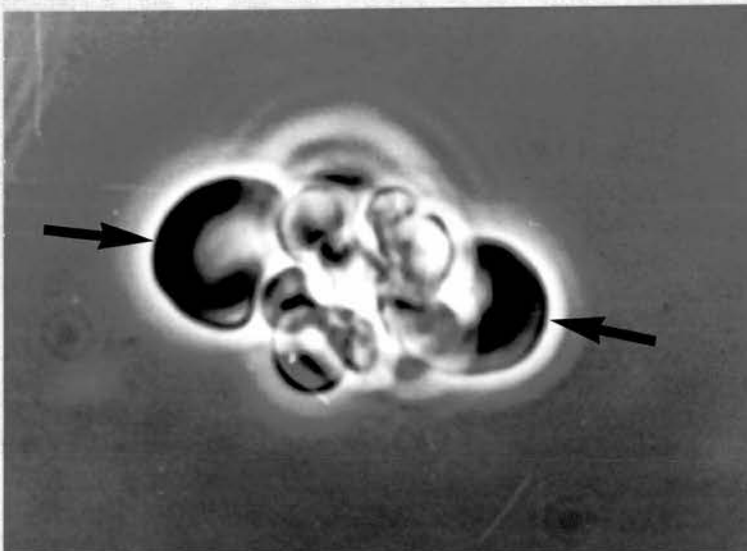


Figure XXVIII X 1,600 This is a mixed sheep and homologous (arrowed) rosette from a patient with A.I.H.A. (Phase contrast).

Cytocentrifuged preparations in A.I.H.A., showed that the majority of rosettes were formed by small lymphocytes (Fig. XXIX). The types of cell forming similar rosettes in glandular fever is shown in Table XIV. More were formed by normal than atypical lymphocytes. Atypical lymphocytes formed rosettes which appeared firmer and tighter than those associated with small lymphocytes.

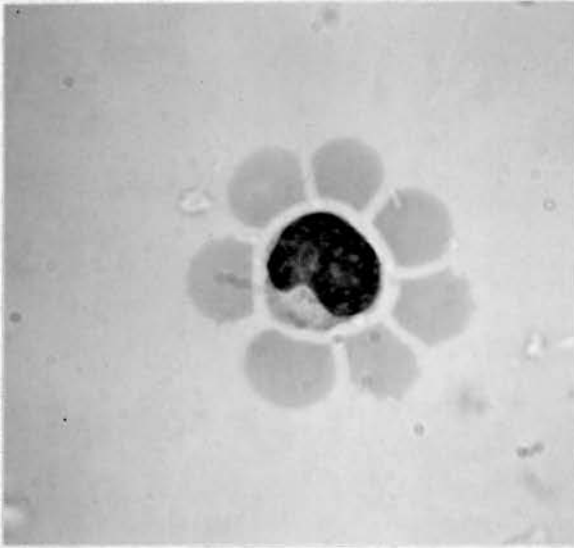


Figure XXIX x 1,200 This is a homologous rosette from a patient with A.I.H.A. (Cytocentrifuge preparation). Giemsa stain.

TYPE OF ROSETTE	TOTAL NUMBER OF ROSETTES	NUMBER AND % OF NORMAL LYMPHOCYTES	NUMBER AND % OF ATYPICAL LYMPHOCYTES
HOMOLOGOUS	164	112 (68.3%)	52 (31.7%)
AUTOLOGOUS	319	233 (73%)	86 (27%)

Table XIV Morphological identification of the rosette forming cell in glandular fever.

ii) Light Microscopy of Phytohaemagglutinin stimulated rosettes

All light microscopic examinations were of phase contrast preparations of 72h cultures. The R.F.C. were large and contained refractile cytoplasmic vesicles which often overlay the nucleus (Fig. XXX). The form of rosette varied and often lymphocytes with a 'hand mirror' appearance formed a 'polar rosette', leaving the uropod free. More frequently the cell was spherical, with varying numbers of red cells and occasionally morulae were formed. P.H.A. induced rosettes had more attached red cells than those seen in similar preparations in A.I.H.A. or glandular fever.

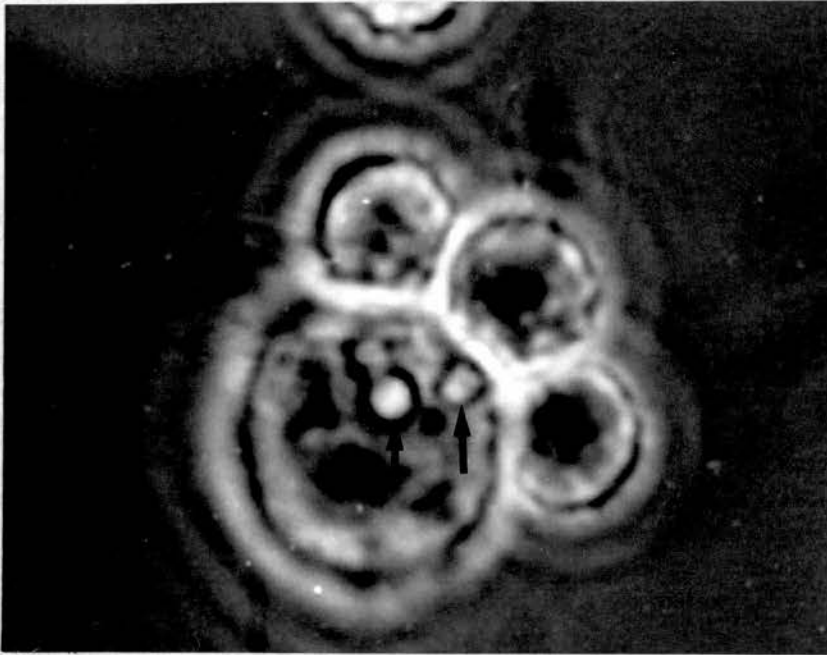


Figure XXX x 1,600 Phase contrast photograph
of a phytohaemagglutinin stimulated rosette
The multivesicular bodies are arrowed.

In the phase contrast preparations of all types
of rosette in the three conditions discussed,
(A.I.H.A., Glandular Fever and P.H.A.), disintegration
of the rosette occurred after 1 hour at room
temperature.

8. Investigation of the physical factors affecting homologous and autologous R.F.C. formation

a) Red Cell Age

With both homologous and autologous preparations no significant differences were noted when old red cells (up to 30 days) were used. These experiments were carried out in normal control subjects only (Figs. XXXI, XXXII).

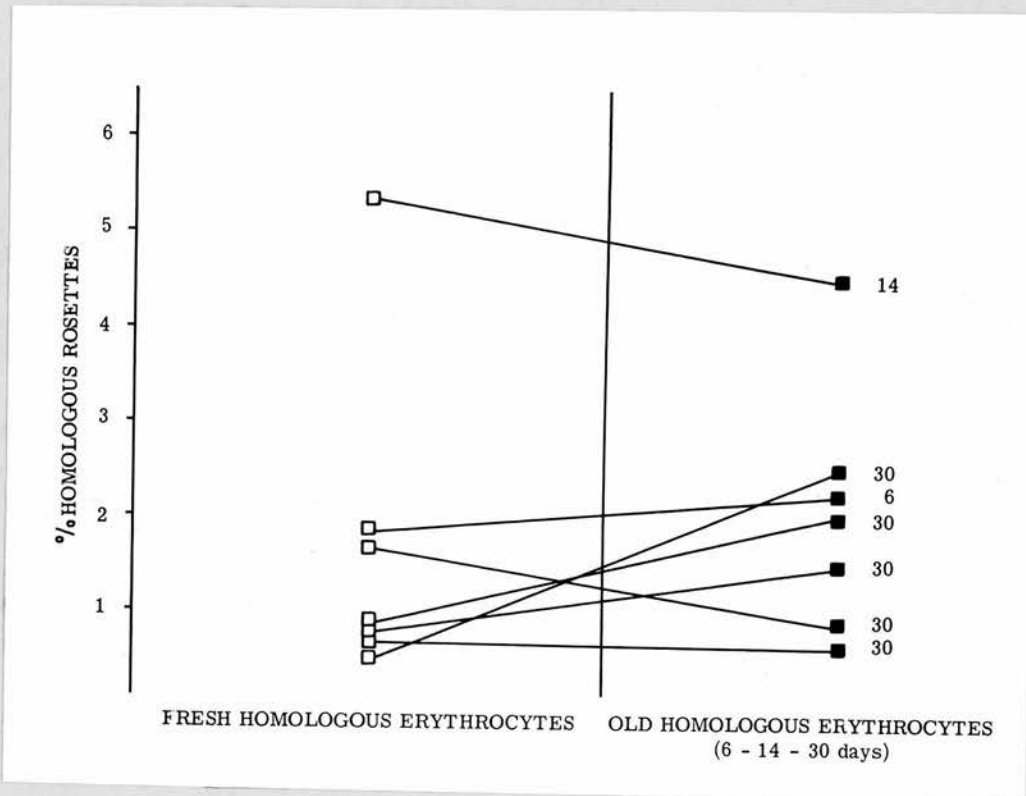


Figure XXXI Red cell age and rosette formation.

This figure shows that the age of the red cell used as the homologous cell has little effect on the number of rosettes formed.

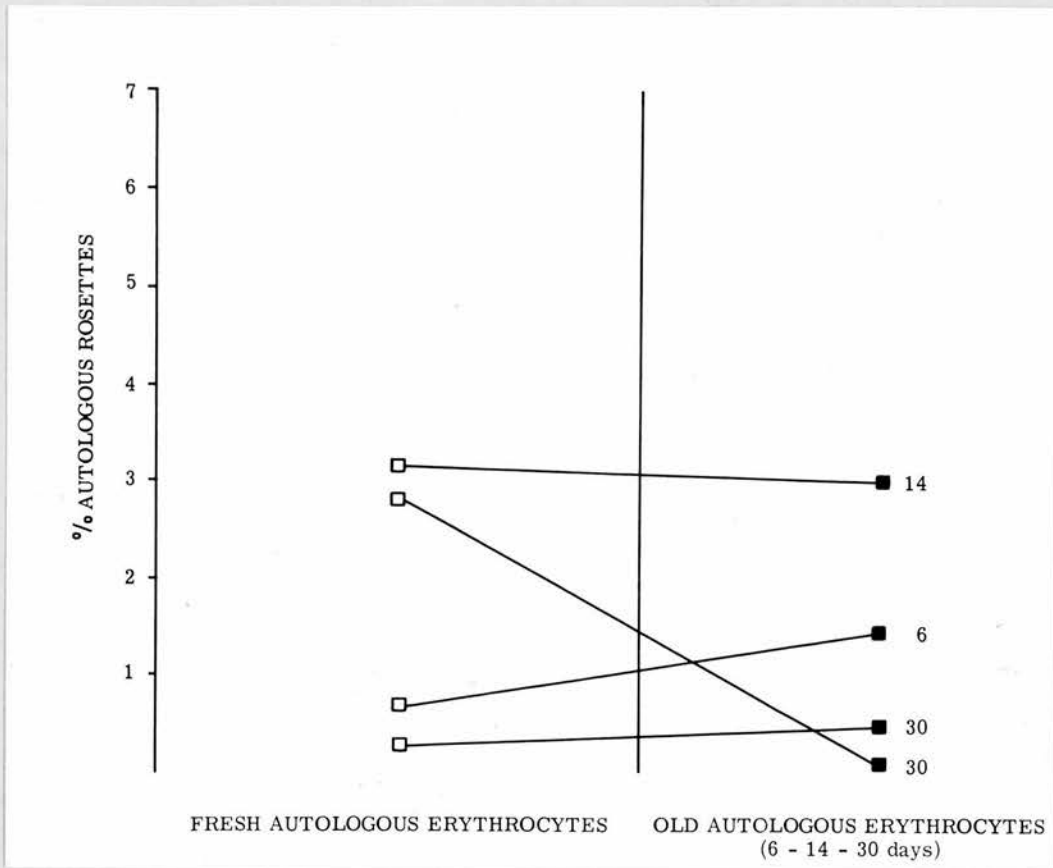


Figure XXXII Red cell age and rosette formation

This figure shows that the age of the red cell used as the autologous cell has little effect on the number of rosettes formed.

b) Supplementation of incubation medium with bovine serum albumin (B.S.A.)

In 6 cases, all with A.I.H.A., the addition of B.S.A. resulted in increased homologous-R.F.C. and had little effect on sheep rosetting cells. A typical example is shown in Figure XXXIII.

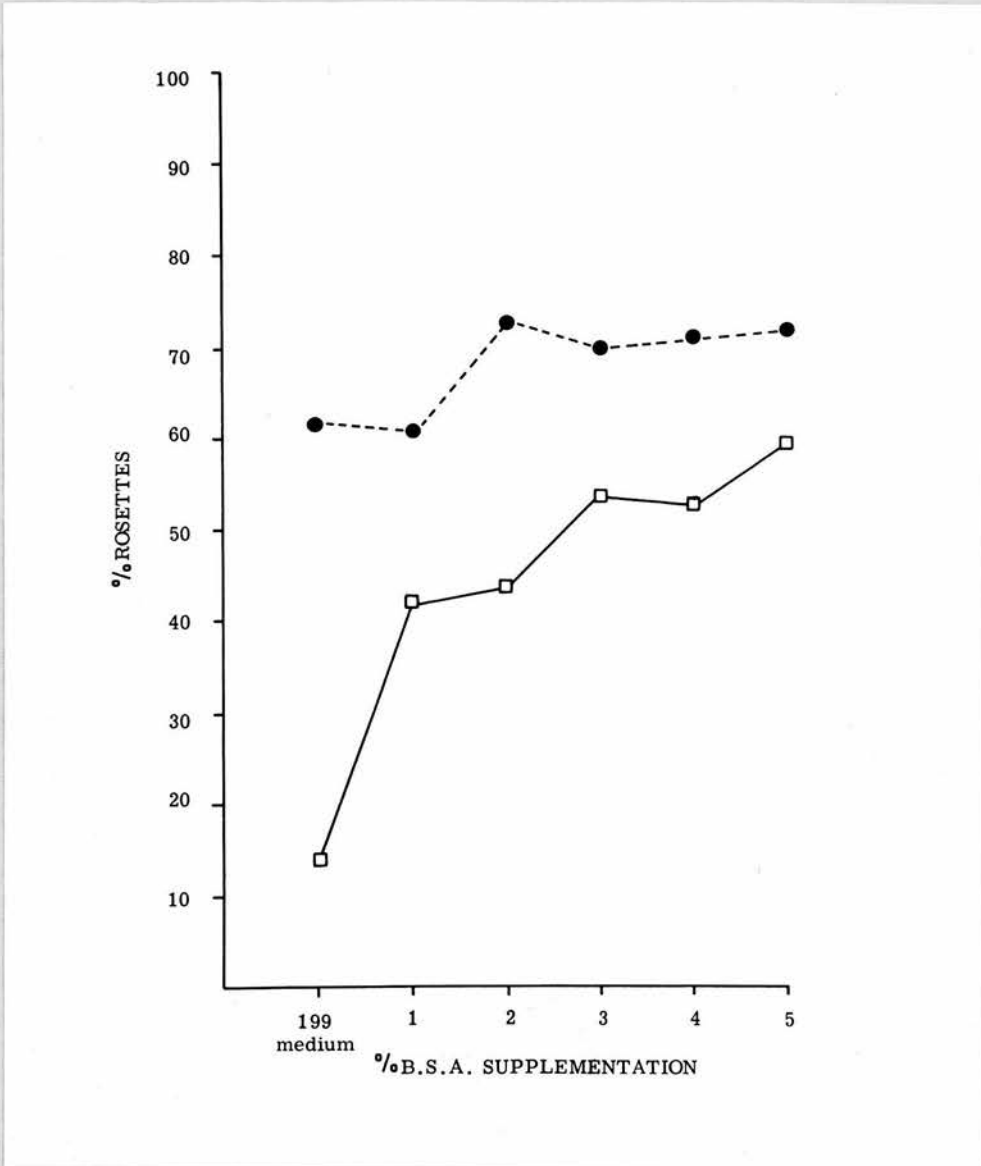


Figure XXXIII The effect of bovine serum albumin supplementation on rosette formation

An increase is seen only in homologous rosettes.
 (□ - homologous rosettes; ● - sheep cell rosettes)

c) Supplementation of incubation medium with human serum

No effect was noted, with the addition of increasing concentrations of AB serum, on the number of rosettes formed by mononuclear cells from thymus and spleen (Fig. XXXIV).

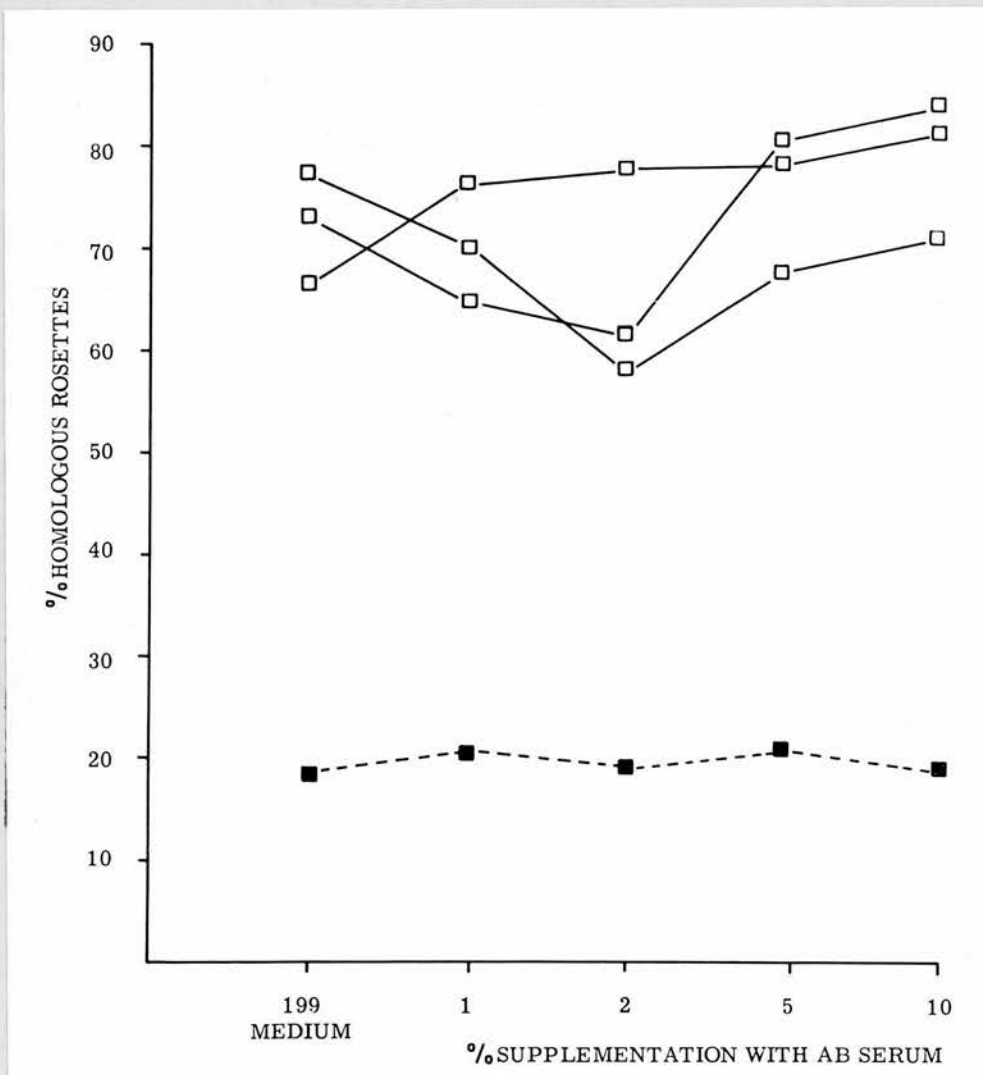


Figure XXXIV The effect of human AB serum on the formation of homologous rosettes. (□ - Fresh thymus cells; ■ - day old spleen cells from patient with A.I.H.A.).

d) The Erythrocyte: White Cell ratio

Lowering the ratio below 40/1 had a depressant effect on rosette number (Fig. XXXV). The demonstration of a plateau effect was not possible, as higher levels of red cells made counting impossible.

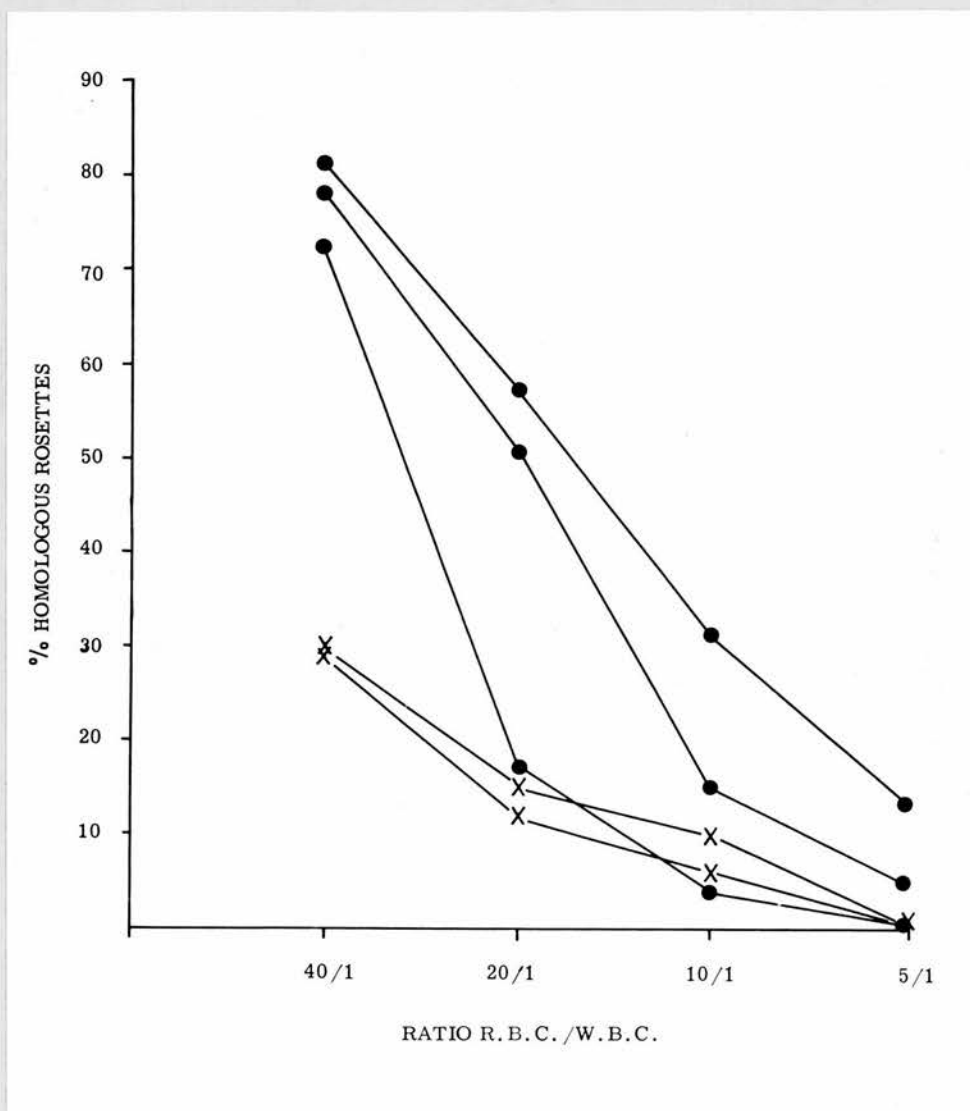


Figure XXXV The effect of the erythrocyte/white cell ratio on rosette formation

Clearly shown is the fall off in homologous rosettes as the ratio drops (● - Thymus cells; (P.M.) x - Peripheral blood cells from patient with A.I.H.A.)

e) Effect of temperature

The number of rosettes fell rapidly with increasing temperature. If left at 37°C for a period exceeding 30 minutes the rosettes disintegrated completely (Fig. XXXVI).

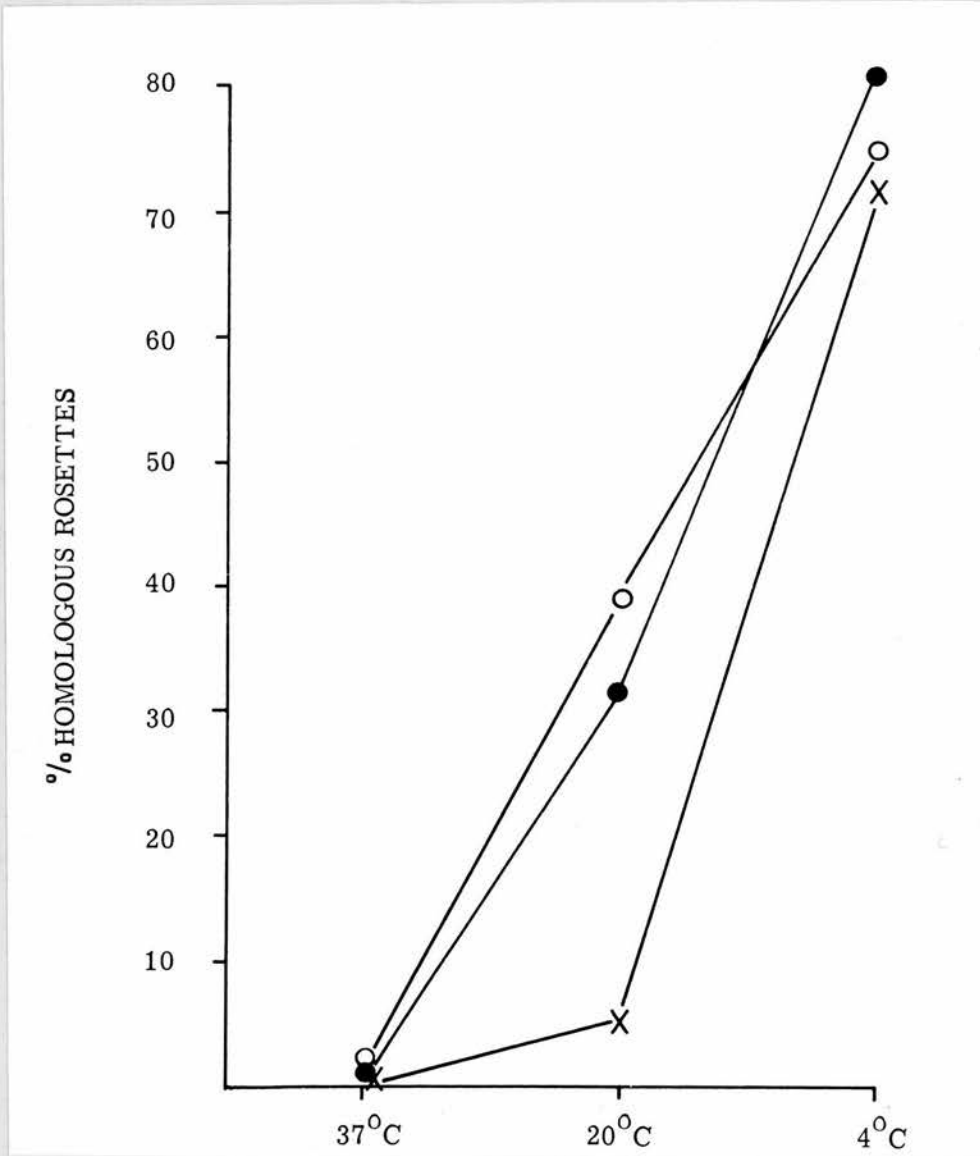


Figure XXXVI The effect of temperature on homologous rosette formation.

Three separate experiments are shown. ● ○ X

f) Effect of Rh-null erythrocytes

Four patients with A.I.H.A. were investigated in this way (Fig. XXXVII). In all four the use of Rh null erythrocytes resulted in a fall in the number of homologous R.F.C.. A similar, and in two cases greater reduction was seen, when red cells of normal genotype, stored in the same manner as the Rh-null cells (-190°C) were used.

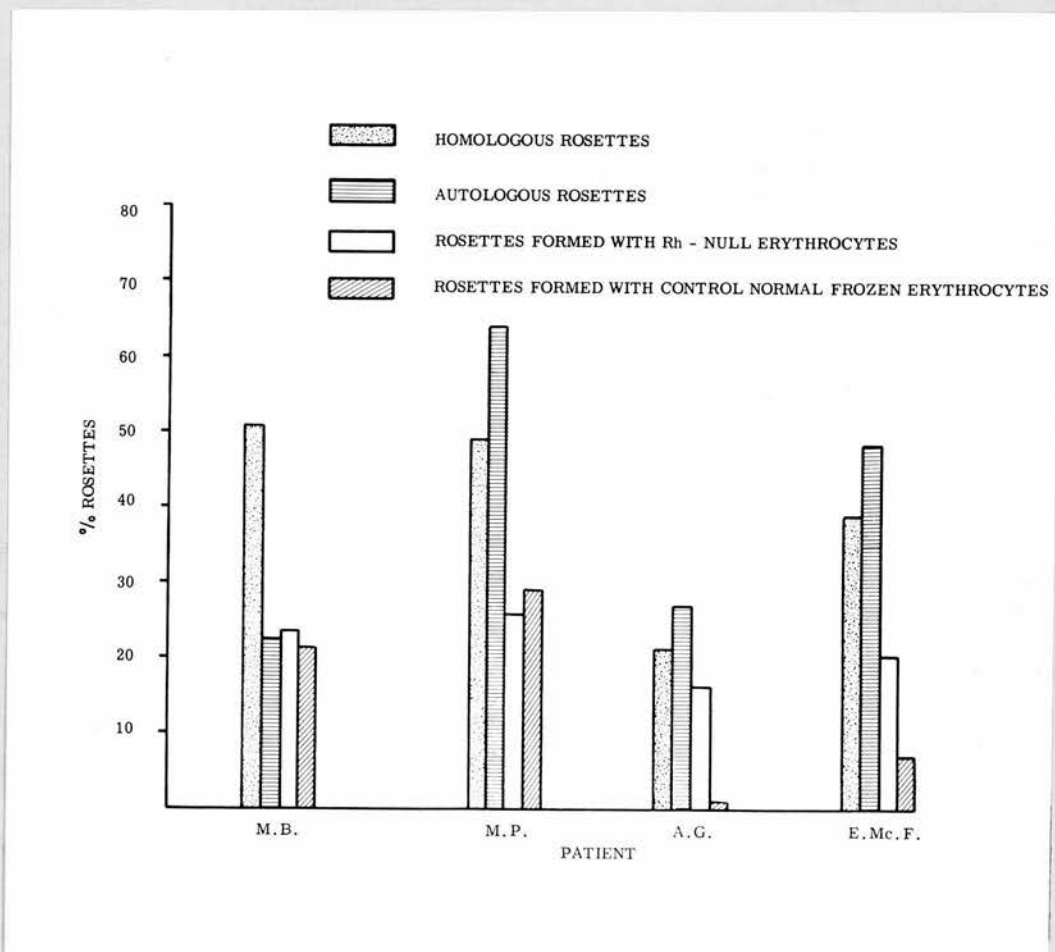


Figure XXXVII The use of Rh-null cells as the

homologous cell.

In all cases the fall caused by the use of these erythrocytes is equalled or exceeded by utilising control frozen erythrocytes.

g) Homologous and Autologous Serum

In all combinations (Fig. XXXVIII) the introduction of such sera to peripheral blood rosette preparations resulted in a depression of rosette number.

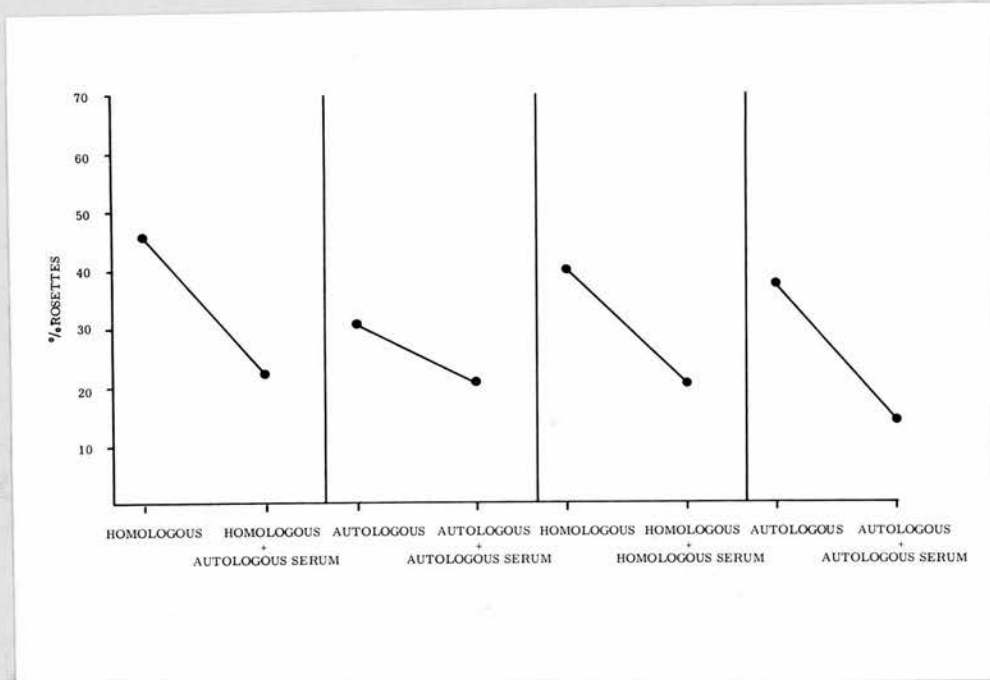


Figure XXXVIII The effect of homologous and autologous serum on rosette formation.

In all cases shown, there is a fall in the number of rosettes.

h) Effect of abnormal red cells

Erythrocytes with various abnormalities were used as the homologous erythrocyte in control personnel. There was no elevation in the percentage of rosettes encountered and further, in/

in the patients themselves, autologous rosettes were within the normal range (Table XV).

AGE OF PATIENT	DISORDER	HOMOLOGOUS ROSETTES %	AUTOLOGOUS ROSETTES %
22	RED CELL HEXOKINASE DEFICIENCY	3.7	2.3
48	RED CELL HEXOKINASE DEFICIENCY	2.0	3.7
25	SPHEROCYTOSIS	0.6	1.4
25	METHYL-DOPA INDUCED HAEMOLYSIS	1.2	2.0
74	PAROXYSMAL NOCTURNAL HAEMOGLOBINURIA	2.8	3.2
73	MECHANICAL HEART VALVE HAEMOLYSIS	3.6	N.D.
57	SIDEROBLASTIC ANAEMIA	0.2	0.3
83	SIDEROBLASTIC ANAEMIA	0.3	0.9

Table XV The effect of abnormal red cells.

Homologous rosettes were measured with the patients erythrocytes using white cells from control personnel. Autologous rosettes were measured using the patients own leucocytes.

DISCUSSION

A. Correlations of levels of homologous and autologous R.F.C. with disease activity

- i) Correlation with disease activity in A.I.H.A..
- ii) Lack of correlation with antiglobulin titre in A.I.H.A..
- iii) Comparison of levels of homologous and autologous rosettes in A.I.H.A..
- iv) Episodes of lymphopenia in A.I.H.A..
- v) Rosette numbers in lymph nodes and spleen in A.I.H.A..
- vi) Level of homologous and autologous R.F.C. in glandular fever and their correlation with disease activity.
- vii) Reversal of T - B cell ratio.

B. Nature of the rosette forming cell

- i) Phytohaemagglutinin transformation
- ii) Identification of the rosetting cell
- iii) Light microscopic features
- iv) Physical factors affecting rosette formation
- v) Conclusions

DISCUSSION

Of fourteen patients with A.I.H.A. which was clinically active, twelve had at some stage, increased numbers of homologous and autologous R.F.C. in the peripheral blood when compared with controls. The preliminary results were therefore confirmed.

Additionally, the findings reported in this study allow answers to be given to the other two questions posed. The first was whether a relationship existed between the activity of the disease, based on clinical and routine criteria, and the level of homologous and autologous R.F.C.. The second concerned the nature of the rosetting cell, primarily with regard to its identification as T or B lymphocyte. These problems are now discussed in two main sections.

Although the number of patients studied was small the results allow certain other general conclusions to be reached. These are discussed in the most relevant main section.

A. Correlations of levels of Homologous and Autologous R.F.C. with disease activity

i) Correlation with disease activity in A.I.H.A.

In nine of the fourteen patients studied
(two/

(two thirds of the total) correlation was demonstrated between the rosette level in the peripheral blood and fluctuations in the disease process, either spontaneous or therapeutically induced (Groups I, II, V). As the disease worsened the rosette level rose and as remission was achieved the levels returned to control values. A critical appraisal of the effect of steroid therapy was not possible because of the small number of patients involved and variability of dosage employed. Splenectomy in four cases (Group I) was followed by a rapid fall in both homologous and autologous R.F.C. to control levels, followed by a pronounced and sustained increase. This feature was not seen in the other two patients who underwent this operation, T.K. (Group V) and A.W. (Group IV).

In the remaining five patients two separable groups were discerned. Group IV demonstrated that quite dramatic changes in clinical condition were not always accompanied by a fluctuation in homologous or autologous R.F.C.. In Group III exactly the opposite occurred. Thus the patient remained clinically in status quo whilst transient elevations of homologous and autologous R.F.C. took place.

The/

The contention that the peripheral blood levels of homologous and autologous R.F.C. reflect disease activity is strongly supported by the low levels identified in most of the patients with quiescent A.I.H.A..

In subjects observed over a prolonged period, the increase in peripheral blood rosetting cells was sometimes sustained but often sporadic. In A.I.H.A. therefore a single determination of rosetting cells does not reliably indicate the activity of the disease, and it is only with continuous observation that a discernable pattern is likely to emerge in an individual patient. This has been demonstrated in two thirds of the subjects in this study. This form of investigation is therefore unlikely to be useful as a diagnostic or prognostic test in its present form.

ii) Lack of correlation with Antiglobulin titre in A.I.H.A.

No relationship was identified between the direct antiglobulin test titre and rosette level, either in individual patients or between different patients, unlike the findings of Gluckman (1970).

Perhaps/

Perhaps of more significance would have been a correlation of the number of rosettes with the results of the indirect antiglobulin test. The latter is more often positive in patients severely ill than in those in comparative remission (Dacie, 1962). No such relationship was found.

iii) Comparison of levels of Homologous and Autologous rosettes in A.I.H.A.

Though discrepancies between the levels of homologous and autologous rosettes were noted in three patients (A.G., M.B., I.W.), in the others there was close agreement between these two forms of R.F.C.. One reason for discrepancy could be experimental error, but why it was seen in only three patients and reasonably consistently in two is unclear. Perhaps the two forms of rosette are due to different mechanisms and one would expect this to be the case in terms of current immunological dogma.

Autologous red cells, apart from one or two exceptions, were coated with complement components or immunoglobulin or both in the experiments described. As the titre of the antiglobulin test correlates reasonably well with the amount of each component sensitising the red cell (Fischer et al., 1974), in some cases autologous erythrocytes were/

were quite heavily sensitised and should have formed rosettes with monocytes and B lymphocytes. These forms of white cell have membrane receptors for IgG and C (Huber and Fudenberg, 1970; Fröland and Natvig, 1973). Low autologous R.F.C. occurred in many cases in which there were high titre antiglobulin tests due to both IgG and C.

The explanation in the case of monocytes could be due to the sub-specificity of membrane receptors, in the case of this cell, they are for C3b and IgG1 and IgG3 (Griffin et al., 1975; Huber et al., 1971).

In A.I.H.A., complement on the erythrocyte is in the form of C3d, in the majority of cases (Engelfriet et al., 1974), and red cells so sensitised would not therefore be expected to adhere to monocytes.

It is possible that the subclass of IgG could play a similar role, but this seems less likely as IgG sensitisation of the erythrocyte is responsible for its in vivo destruction by macrophages. With red cells sensitised with IgG2 and IgG4 destruction by macrophages should not theoretically take place, and thus haemolytic disease should not exist. However, there is no information of the subclass of IgG which makes up the auto-antibody in A.I.H.A. and no definite conclusions can be reached on this point./

point.

The lack of interaction of similarly sensitised autologous red cells with B lymphocytes is quantitatively much more important, but difficult, and at the present state of knowledge, impossible to explain. Subclass specificity of the Fc receptor on B lymphocytes is not known though there is lack of reaction with IgA and IgM in the mouse (Basten et al., 1972).

A possible mechanism is that auto-antibody in A.I.H.A. produced in vivo, may in addition to sensitising autologous erythrocytes, adhere to autologous B lymphocytes which have Fc receptors (Dickler and Kunkel, 1972; Fröland et al., 1973; Brain and Marston, 1973), thus acting as a blocking antibody.

This may be too simplistic an approach but the evidence of Gluckman et al. (1974) suggests such a mechanism could play a part. These authors showed that antigen sensitive cells in rats were suppressed in tolerant animals and that this suppression was most likely due to serum factors inhibiting recognition of the tolerated antigen.

Such blocking mechanisms have been shown to exist in other systems in rats for autoantigens (Wekerle et al., 1973), for tumour enhancement (Hellström and Hellström, 1970) and for self tolerance/

tolerance in the mouse (Micklem, 1971). The nature of the molecule responsible has not been definitely identified but in the various publications quoted, soluble antigens, antigen-antibody complexes or antibodies have been proposed.

Another possibility is that the scarcity of Rh sites found on the erythrocyte (Nicolson et al., 1971) limits the surface density of antibody. This limited degree of sensitisation might affect in vitro erythrocyte adherence to B lymphocytes via Fc receptors in some cases.

The experimental conditions themselves are unlikely to have been responsible for the low autologous R.F.C. with sensitised erythrocytes, as it has been shown that Fc aggregate binding is independent of temperature, time or the presence of protein in the medium (Dickler and Kunkel, 1972).

In the case of complement sensitisation, similar limitation by the low density of Rh sites might interfere with efficiency of binding by the central lymphocyte. More recently C3-mediated cytoadherence by lymphocytes has been shown to be temperature dependent (Dierich and Reisfeld, 1975). They suggested that a local increase in C3 receptor density by aggregation was a prerequisite for C3 dependent cytoadherence. This aggregation was inhibited/

inhibited by low temperatures. The low temperature employed in the experiments described in this thesis might be the explanation for lack of complement mediated adherence to lymphocytes, if and when it was required.

iv) Episodes of lymphopenia in A.I.H.A.

Transient periods of lymphopenia occurred in some patients during the course of the disease. In some the lymphopenia was spontaneous, in others it occurred coincidentally with steroid therapy or followed splenectomy. This phenomenon has not been remarked on previously and requires further evaluation in a larger series of patients with A.I.H.A..

v) Rosette numbers in lymph nodes and spleen in A.I.H.A.

The study of tissue, in a limited number of patients, was surprising because of the low level of homologous and autologous R.F.C. in most cases studied. This was despite high peripheral blood values in many at the time of examination (A.G., W.C., M.P.). It seems unlikely that such cells achieve the ability to rosette only when present in the peripheral blood. The series requires extension and an interesting investigation would/

would be examination of the bone marrow for similar cells in such patients.

vi) Level of homologous and autologous R.F.C. in glandular fever and their correlation with disease activity

Investigations were carried out in patients with glandular fever because in a significant proportion of cases auto-antibodies are produced as a feature of the disorder (Leader, 1973). Rarely major haemolysis, but more often minor degrees of haemolysis due to auto-immune processes can be detected (Dacie and Worrlidge, 1969). It is possible that similar mechanisms to those underlying the pathogenesis of glandular fever, are operative in the initiation of cases of idiopathic auto-immune haemolytic anaemia. Thus evaluation of the former, on a cellular basis, might add to and extend information derived from patients with A.I.H.A..

The results showed, that in all patients studied, homologous and autologous R.F.C. were elevated in the acute phase of the disease. In two patients studied sequentially, both the homologous and autologous rosettes had returned to their normal values within one month.

The/

The transient fall in the rosetting population following steroid therapy seen in one patient (G.W., Fig. XVII) could be due to extravascular redistribution of T lymphocytes from the bloodstream (Fauci and Dale, 1975). Despite continuing oral steroid in this patient the R.F.C. returned to their previously high levels, indicating that they are not particularly sensitive to this drug.

At the time of this investigation Sheldon and Holborow (1975b) published results showing similar results to mine in glandular fever, and in other disorders involving T cell proliferation.

It therefore seems that elevation of homologous R.F.C. (and autologous R.F.C.) is characteristic of glandular fever in the acute phase. Because of normal wide variation in controls, the results of T and B lymphocyte and monocyte numbers are difficult to interpret. The consensus of opinion indicates that atypical lymphocytes in this disorder fall into two main populations, a major T lymphocyte and a minor B lymphocyte component (Denman and Pelton, 1974; Mangi et al., 1974). The results given in this thesis would not contradict this finding, but markers originally described for normal lymphoid cells have been applied to rapidly proliferating, abnormal cell populations and thus interpretation requires caution.

vii) Reversal of the T/B cell ratio

This phenomenon was recorded in two patients with A.I.H.A. (I.H. and T.K.). In one it was an acute process (I.H.) and related to the course of the clinical illness, in the other (T.K.) it was chronic. The most obvious explanation for this effect is that an underlying process, such as infection or an immune challenge, has resulted in a rapid outpouring of B-lymphocytes specifically directed against that challenge. It would be interesting and perhaps rewarding to do sequential peripheral blood T and B lymphocyte levels on further patients with A.I.H.A..

B. Nature of the rosette forming cell

The prime interest of this section was the identification of the lymphocytic rosette forming cell. In particular, it was felt important to establish whether the cell was thymus or bone marrow derived. From the small series of patients with miscellaneous disorders of circulating leucocytes it seems that B lymphocytes and their progeny (plasma cells), monocytes and lymphoblasts, the latter lacking membrane markers, do not form homologous or autologous rosettes to any significant degree. It was also interesting to find that one patient with a chronic lymphocytosis due predominantly to T lymphocytes did not have high levels of rosetting cells. This latter finding indicates that an elevation of T lymphocytes per se does not result in high homologous or autologous rosettes. Attention was then directed primarily to in vitro investigation.

i) Phytohaemagglutinin Transformation

Support for the T cell nature of the rosetting lymphocyte was obtained from studies with phytohaemagglutinin (P.H.A.) stimulation. Although there is a report of transformation of B lymphocytes by this mitogen (Phillips and Roitt, 1973), most authors consider it to be primarily a T cell stimulant/

stimulant (Greaves et al., 1974b; Mendes et al., 1974). The results in this thesis showed that homologous and autologous rosetting cells were consistently increased in P.H.A. stimulated cultures. Other workers have shown an increase in homologous rosette formation in such cultures (Sheldon and Holborow, 1975a), and Blecher (1974) has shown an increase in auto reactivity in similar experiments.

The mechanism leading to the increase of homologous and autologous rosettes could be due to a change, or an increase in surface receptors in stimulated cells. It has been shown (Weksler, 1973) that lymphoblasts, resulting from incubation with P.H.A., stimulate autologous lymphocytes to transform. The author postulated the appearance of new determinants on P.H.A. treated lymphocytes. Secondly, non-specific activation of monocytes results in increased binding sites for IgG (Arend and Mannik, 1973).

Less specific factors may play a part, thus P.H.A. stimulation has been shown to result in an increase, both in number and length of villous processes by the transformed lymphocyte (Clarke et al., 1971; Jerrells and Hinrichs, 1975).

The formation of such villi or small pseudopodia probably results in a reduced local charge density in/
in/

in the vicinity of the tip of the process, which reduces mutual electrostatic repulsion between cells and encourages adhesion (Bangham, 1964). Such a non-specific mechanism might therefore play some part in the development of homologous and autologous rosettes in P.H.A. cultures.

Certainly, movement would appear to be essential, as Cytochalasin-B prevented the increase in rosetting cells. This drug clearly inhibits cell movement but its site of action is controversial. It is thought to act on contractile proteins and microfilaments causing their disruption (Unanue and Karnovsky, 1974), such elements are concerned with cell movement (Spooner et al., 1971). Inhibition of cell movement and consequently the ability to form cellular processes, could explain the inhibition of homologous and autologous R.F.C. formation in the P.H.A. stimulated system.

ii) Identification of the rosetting cell

Two techniques were used to identify the nature of the rosetting lymphocyte. Prior treatment of cell suspensions with anti-human immunoglobulin (F.I.T.C.) reagents, followed by the preparation of rosettes, showed that the majority of rosettes both in A.I.H.A. and glandular fever did not carry surface immunoglobulin. Cells in man with easily demonstrated/

demonstrated immunoglobulin are B lymphocytes (Fröland and Natvig, 1973). Two factors should be considered when interpreting these results. First cells actively secreting immunoglobulin (Ig) do not carry surface Ig determinants (Paraskevas et al., 1970). Secondly 'null cells', which lack any surface markers and which have been extensively investigated in mice (Stobo et al., 1973), have recently been shown to exist in man (Chess et al., 1975). The relationship of 'null cells' to T and B lymphocytes and to monocytes is unresolved, though Chess et al. (1975) have claimed that such null cells, after three days in culture, develop surface immunoglobulin.

Therefore the results with the fluorescent preparations do not exclude 'null cells' nor do they exclude Ig secreting cells. 'Null cells' however, lack receptors for sheep erythrocytes which are found on T lymphocytes (Bach et al., 1969). The results of the mixed rosetting showed that in A.I.H.A., almost all the homologous rosettes had receptors for both sheep and human cells. In glandular fever almost a similar picture obtained, though in an occasional patient (M.D.) high numbers of homologous rosettes alone were found. A reasonable conclusion would appear to be, that in most patients, the central rosetting cell was a/

a T lymphocyte.

Two other features were noted in the mixed rosetting experiments and require comment. On the majority of occasions the values of mixed rosettes were higher than for homologous and autologous rosettes. A possible explanation for this phenomenon is the observation of Lin and Wallach (1974) who found that cells rosetted with sheep erythrocytes developed short microvilli in the region of red cell contact and with increasing time these microvilli became long, undulating and branched. The development of such processes would, if the previous argument proposed in the section on P.H.A. culture is correct, result in an increased propensity for rosetting.

Secondly, on every occasion the sheep erythrocyte rosettes measured by Wigzell technique were higher than by the conventional method. The most likely explanation is that the increased mechanical disruption employed in the conventional technique, broke the more fragile rosettes which were detected by the gentler Wigzell method.

iii) Light microscopic features

The majority of rosettes in A.I.H.A. and glandular fever were small lymphocytes. Consistently fewer red cells were involved in the/

the formation of homologous and autologous rosettes than in sheep cell rosettes. This was shown by phase contrast of living preparations and fixed preparations. Prior treatment of the rosetting cells with glutaraldehyde, a procedure claimed to prevent disruption (Haskill et al., 1972), did not result in higher numbers of attached erythrocytes. The bulkiness of the human erythrocyte, as compared to the sheep erythrocyte, might explain the difference of number of attached red cells. Additionally it might be due to a different mechanism of binding of the two forms of red cell.

It is now recognised that T lymphocytes bind considerably less antigen than B lymphocytes and that such binding by T lymphocytes is more fragile. (Bach, 1973 a; Haskill et al., 1972; Hogg and Greaves, 1972). The human T lymphocyte usually binds seven or eight sheep cells (Bach, 1973 b) and the specific antigen binding cells which are thymus derived bind less than ten sheep cells (Haskill et al., 1972). Thus the light microscopic results show, that in terms of antigen binding and apparent lack of affinity, the homologous rosetting cells have the properties of T lymphocytes.

iv)/

iv) Physical factors affecting rosette formation

The effect of some physical variables on homologous and autologous rosette formation is discussed here, Bach (1973b) has recently reviewed the physical factors which influence sheep erythrocyte rosette formation.

Erythrocyte membrane changes consequent to ageing were without effect, with regard to the production of homologous or autologous rosettes. Additionally in the last experiment, it was recorded that the use of erythrocytes, derived from patients with various non-immune haemolytic disorders, did not increase homologous (donor leucocytes), or autologous rosettes. Most of the latter conditions are associated with a membrane defect which has not yet been precisely defined.

Therefore surface changes in the membrane of the erythrocyte, certainly due to the causes enumerated above, do not of themselves lead to increased homologous or autologous rosette formation. Micklem and Asfi (1970) have postulated that in the mouse, autologous rosetting cells might be related to phagocytosis and disposal of aged autochthonous erythrocytes. The results I have presented here would not support such a function for homologous and autologous rosetting cells in humans.

Homologous and autologous R.F.C. formation was exquisitely temperature sensitive and rosettes soon disintegrated/

disintegrated even at room temperature. Further, the ratio of lymphocyte to erythrocyte in the initial suspension was extremely important, a feature of sheep erythrocyte rosette formation (Bach, 1973b), and homologous rosettes in the mouse (Laskov, 1968; Micklem and Anderson, 1972).

In several ways lymphocyte binding of human erythrocytes resembles T lymphocyte binding. Thus thymus derived rosettes are much more fragile than B lymphocyte rosettes (Schlesinger and Galili, 1974). The reason for this fragility could be related to receptor affinity, density, turnover or shedding on contact with antigen, or indeed to some non receptor property of T lymphocyte membranes.

The results of bovine serum supplementation are in accord with the report of Yu (1975) who showed a similar increase in homologous rosettes.

The results concerning the antigen specificity of homologous rosettes showed that when frozen stored Rh-null erythrocytes were used, the number of rosettes fell, but a similar and in two cases greater fall occurred with stored, frozen normal erythrocytes. Thus, the conclusion is, that a proportion of homologous rosettes do not appear to be dependent upon the presence of Rh-antigen on the red cells used. It would be of great interest to use fresh Rh-null cells in this system especially in/

in a patient where quite clearcut specificity of the antibody for the Rh-system could be demonstrated.

Reduction of rosettes occurred in all situations where the various types of sera were used in controls and tests. This would suggest the effect was non-specific, in no case were the elevated rosettes reduced to control levels.

v) Conclusions

In conclusion, both homologous and autologous R.F.C. have been shown to be significantly elevated in many cases of clinically active A.I.H.A. and in about two thirds to show correlation with the disease activity. Investigation has shown that rosettes with similar properties can be demonstrated in the acute phase of glandular fever and in lymphocyte cultures which have been stimulated with P.H.A.. Many of the other investigations reported here suggest that a large proportion of these cells are thymus derived. In Chapter V there will be a discussion on the role these cells might play in the pathogenesis of A.I.H.A..

CHAPTER IV

ULTRASTRUCTURAL STUDIES
OF THE ROSETTE FORMING
CELL IN AUTO-IMMUNE
HAEMOLYTIC ANAEMIA,
GLANDULAR FEVER AND
PHYTOHAEMAGGLUTININ
STIMULATED CULTURES

1. Plan
2. Immune rosettes in animals
 - i General description
 - ii The binding sites
3. Immune rosettes in man
 - i General description
 - ii The binding sites.
4. Ultrastructure of non-immune rosetting cells
5. Ultrastructure of lymphocytes
 - i General description
 - ii Ultrastructure of B cells
 - iii Comparison of the ultrastructure of T and B cells.
6. Ultrastructure of monocytes and their interaction with red cells
 - i The monocyte
 - ii Interaction of monocytes with red cells
 - iii Comparison of monocyte and lymphocyte interaction with erythrocytes.
7. PHA transformation of lymphocytes as observed by transmission electron microscopy
8. The ultrastructure of the K-cell
9. Scanning electron microscopy of rosette forming cells
10. Design and purpose of present investigation.

1. Plan

The purpose of this chapter is to describe in detail the ultrastructural features of the rosetting cell, together with the nature of its interaction with adherent erythrocytes. Before the results, a review of the relevant literature is given to provide a suitable background for interpretation.

The sections into which the review is divided correspond to the major questions which were posed and which are delineated in the discussion. The first section describes the ultrastructural features of immune and non-immune rosettes in animals and man. This is followed by a detailed description of T and B lymphocytes and ultrastructural features which are claimed, in recently published work, to distinguish the two.

The ultrastructure of monocytes and their interaction with sensitised red cells forms the next section. Following this, three separate topics are described, the ultrastructure of phytohaemagglutinin transformed lymphocytes, killer cells (K-cells), and finally recent publications concerning scanning electron microscopic studies of rosetting cells.

2./

2. Immune rosettes in animals

i) General description

A definition of such rosettes has been given in the previous chapter. Their ultrastructure was first described in the guinea pig (Storb et al., 1967) and mouse (Storb et al., 1969). In the guinea pig four types of R.F.C. were observed, lymphocytes, plasmacytes, blast cells and macrophages. The lymphocytes were either small, with sparse cytoplasm containing few organelles apart from many free ribosomes, or large, in which rough endoplasmic reticulum, mitochondria and many free ribosomes were present. The blast cells were characterised by a large vesiculated nucleus and the presence of polyribosomes in the cytoplasm. The plasma cells were identified by their extensive profiles of endoplasmic reticulum, and macrophages by their low nuclear-cytoplasmic ratio and high numbers of cytoplasmic organelles.

In the mouse ninety-four immune rosette forming cells were examined (Storb et al., 1969). Thirty-six were small or large lymphocytes which had features similar to those in guinea pigs. Eight were plasma cells, nine cells were difficult to classify, having abundant cytoplasm and a round or ovoid nucleus which contained loose chromatin. Seventeen/

Seventeen cells were distinguished by the presence of large, spheroidal cytoplasmic bodies, containing dense, finely granulated material bounded by a single membrane. The remainder were macrophages. The high number of macrophage rosettes seen, was thought to be due to their resilience during isolation of the individual rosettes.

Other workers have found, that during the rapid increase in specific R.F.C. following immunisation that two peaks of such cells (days three and six) could be identified in the antibody-producing mouse spleen (Gudat et al., 1970; Gudat et al., 1971). The most likely explanation for the biphasic curve was the production of IgM followed by IgG, as such a feature was absent from the secondary response.

The R.F.C. examined at three days had a relatively narrow rim of cytoplasm containing dense ribosomes, occasionally in clusters, with increased mitochondria and occasional short profiles of endoplasmic reticulum. Nuclear chromatin in these cells was loose and nucleoli were prominent. On the sixth day there was more advanced maturation of the cytoplasm, with increased volume and content of mitochondria and poly-ribosomes. Cells incorporating thymidine were predominantly lymphocytes but/

but there were some well differentiated plasmablasts as well.

The conclusion that can be drawn from these results, is that rosetting cells, which arise following specific immunisation, have a heterogeneous make-up. They range from small lymphocytes to plasma cells. Macrophages are well represented in such populations. In none of the above studies was mention made of macrophage deformation of the attached red cell.

ii) The binding sites

Detailed studies, of antigen binding sites of immune rosetting cells from mice, using scanning and transmission electron microscopy have recently been published (Gudat & Villiger, 1973). Results with both techniques showed that adherence of the erythrocytes was mediated by circumscribed binding areas on the lymphocyte surface. The binding sites varied from small point-like, to broad contact areas. An average distance of 8 nM was found between the white and red cell membranes and some electron-opaque bridges were observed between the membranes of such close contacts. A further observation was an increase in electron density along the inner aspect of the lymphocyte cell membrane at some points of adhesion./

adhesion. Immunoglobulin was present around these spot-like areas, as shown by ferritin labelled antibodies.

A similar pattern of adherence in immune rosettes was noted by Storb et al. (1969). 'Close-fit adherence' was the name given by the authors to attachment of a broad area of red cell membrane. In others, the contact was between a small area of the lymphoid cell and a small protuberance from the red cell surface ('point-adherence'). 'Close-fit adherence' was found in almost all macrophage rosettes but in lymphocyte rosettes point adherence predominated.

3. Immune rosettes in man

i) General description

An opportunity to study immune rosettes in man, was afforded by the discovery of rosette forming cells with Rh-positive erythrocytes amongst the circulating leukocytes of two pregnant, iso-immunised females (Elson & Bradley, 1970). In these subjects no R.F.C. were identified with Rh-negative erythrocytes. In an extension of these studies, electron microscopy of such immune R.F.C. was included (Elson et al., 1972). The central mononuclear cells were of lymphocyte-like/

like appearance. There was coarse chromatin along the inner side of the nuclear membrane, the ribosomes were not densely clustered and a few fragments of endoplasmic reticulum were seen.

Recently, R.F.C. from the peripheral blood of patients with cold agglutinin disease have been described (Feizi et al., 1973). Again such cells were predominantly lymphocytes; plasma cells rarely formed rosettes. The lymphocytes had a similar appearance to those described by Elson et al. (1972).

ii) The binding sites

In both these cases, the contact between lymphocyte and erythrocyte was by micro projections from the surface of the lymphocyte. Broad areas of contact were not frequently encountered. In one paper (Elson et al., 1972) the micro projections were markedly diminished by cytochalasin, a substance which disrupts microfilamentous networks and interferes with cell movement (Spooner et al., 1971). Inhibition of formation of these micro projections, at 4°C was postulated as the cause of the fall in the number of rosettes which was noted at low temperatures. Thus the contractile activity of the cell was important for rosette formation in this system.

4. Ultrastructure of non-immune rosetting cells

As only two reports have been published concerning such cells, one in animals and one in man, they will be described together in this section.

R.F.C. can be detected in low numbers in animals which have not been actively immunised to the relevant antigen. For rosette formation, such antigens must be particulate, sheep red blood cells and human red blood cells are two examples (McConnell et al., 1969; Reyes & Bach, 1971). The nature and function of these cells have so far been the subject of only a few reports, and their ultrastructure has been recorded on only two occasions. As this thesis is concerned in large part with the identification and definition of the properties of such cells, these two publications are of particular relevance.

The first report, concerns spontaneous rosette forming cells to sheep red blood cells in mice (Reyes & Bach, 1971). The lymphocytic R.F.C. were small, with a high nuclear-cytoplasmic ratio. They generally had the features of a mature interphase lymphocyte. Thus the cytoplasm had few organelles, apart from ribosomes which were monoribosomal and the nucleus contained clumped chromatin/

chromatin (Dicke et al., 1973). The erythrocyte-lymphocyte contact varied from a small, to a large interface, not many cytoplasmic processes were seen and the surrounding red cells were not deformed. Macrophage forming rosettes were also seen and in these characteristically the red cells were deformed.

The homogeneity of the lymphocyte population in this situation, contrasts with the mixed cell types seen with heterologous erythrocyte immunisation.

The most recent report of non-immune rosette forming cells is that of Levy et al. (1975). They studied the morphological nature of rosette formation, by both phytohaemagglutinin-stimulated and non-stimulated human lymphocytes, with sheep red blood cells. Lymphocytes stimulated by phytohaemagglutinin are predominantly T-cells (Greaves et al., 1974b). The only new finding reported, was an increase in number and lengthening of the binding sites, between sheep red blood cells and the rosette forming cell in the phytohaemagglutinin stimulated cultures. They suggested that these features possibly represented lymphocyte activation, as they were not found in the rosettes of unstimulated cultures.

5. Ultrastructure of lymphocytes

i) General description

Identification of monocytes does not normally present difficulty, though occasionally their distinction from lymphocytes can present problems (Van Furth, 1970). As distinction between T and B lymphocyte in mouse and man (Matter et al., 1972; Levy et al., 1975) on ultrastructural grounds has recently been attempted, it is pertinent to describe briefly the ultrastructure of human lymphocytes in general terms.

The nucleus of the inactive lymphocyte has a coarse chromatin pattern, characteristic of the interphase cell. Electron dense hetero-chromatin is condensed along the inner side of the nuclear membrane and the nucleolus is small. The nucleus is surrounded by a double membrane with an intervening space of 20-40 nM, and this is obliterated at the region of the nuclear pores. Ribosomes can be seen sometimes on the outer nuclear membrane. The cytoplasm contains monoribosomes, a small Golgi apparatus and one or two profiles of endoplasmic reticulum.

The intermediate lymphocyte is a little larger, with a nucleus similar to the cell previously described/

described. The Golgi-area is larger, the ribosomes appear in clusters and are often associated with rough endoplasmic reticulum, sometimes the nucleolus is large. This latter organelle plays an important role in the elaboration of cytoplasmic R.N.A. and in most cells, the size of the nucleolus is roughly proportional to the amount of ribosomal R.N.A. in the cytoplasm (Zucker-Franklin, 1969).

ii) The ultrastructure of B-cells

Numerous groups of workers have studied the ultrastructure of bone marrow derived lymphocytes and have used various techniques to identify them as B-cells. The techniques have included binding of radiolabelled antibody (Perkins et al., 1972; Bosman et al., 1969; Santer et al., 1972), immuno-ferritin labelling (Reyes et al., 1975), isolation of plaque forming cells (Hummeler et al., 1966), reversed immunocytadherence technique (Zucker-Franklin & Berney, 1972), EAC rosette formation (Chen et al., 1972).

All such investigations have confirmed that the B-cell is a lymphocyte, with variable amounts of rough endoplasmic reticulum, most pronounced in plaque forming (i.e., antibody producing) cells (Hummeler et al., 1966). In the human tonsil, it was found that the size, the nuclear-cytoplasmic ratio/

ratio, the amount of rough endoplasmic reticulum or the size of the Golgi apparatus, could not be used to forecast whether any particular cell was likely to bear surface immunoglobulin (B-cell) (Zucker-Franklin & Berney, 1972).

When the mode of identification involved a particulate antigen, such as a red blood cell, rosettes were formed. Thus EAC rosettes in mice (Chen et al., 1972) showed various forms of contact, from finger-like projections from the lymphocyte to the red cell, finger-like projections from the red cell to the lymphocyte, contact of projections from each type of cell to broad areas of contact. The attachments in this system were strong because following papain dissociation, fragments of erythrocytes remained attached to lymphocytes. In studies of human tonsillar rosette formation (Zucker-Franklin & Berney, 1972), at areas of contact between white and red cell, 'bridges' were seen to be formed by antibody. In some sections, such strands or bridges appeared to be in continuity with similar strands in the cytoplasm of the lymphocyte.

A feature of plasma cell rosettes was a multiple layer of erythrocytes aggregated round the central cell and often many processes extending/

extending a considerable distance from the body of the R.F.C. (Zucker-Franklin & Berney, 1972).

iii) Comparison of the ultrastructure of T and B cells

Most studies have depended upon the lack of immunoglobulin on T cells, when compared to B cells, for their identification. Using such criteria, T cells have been shown to have a higher nuclear cytoplasmic ratio than B cells and a less dense cytoplasm, containing many monoribosomes (Perkins et al., 1972).

Human B lymphocytes have been shown to have a villous surface, in contrast to T cells which have a smoother outline (Reyes et al., 1975).

The difference from previous reports, is explained by the authors on the basis that earlier studies were carried out at low temperatures, a process which reduces the number of lymphocyte microvilli.

The only extensive comparison of the ultrastructure of T and B lymphocytes published, is that in the mouse (Matter et al., 1972). Three categories of T cell were identified. T_1 were small to medium sized lymphocytes with a clear cytoplasm, and apart from monoribosomes, contained few organelles. T_2 were large cells with numerous polyribosomes often in a rosette form, and these cells had a developing/

developing filamentous network. T_3 cells were very characteristic. They were dark, small to medium sized lymphocytes, usually containing large amounts of closely packed ribosomes and showed a striking accumulation of filamentous network, often condensed in large areas devoid of cell organelles. T_3 cells were rarely seen in the thymus, but T_2 cells constituted the cortisone resistant medullary thymocytes. These authors suggested that the T_1 lymphocytes were virgin T cells, T_2 activated and T_3 differentiated T cells. Thus, in the mouse, a striking development and dense accumulation of microfilaments appears to be characteristic of activation and differentiation of T cells. Such a filamentous network was not seen in B cells.

A microfilamentous cytoplasmic structure has recently been identified in pathological human T cells often called 'Sezary cells' (Zucker-Franklin et al., 1974). The electron micrographs shown in this paper, though this feature is not specifically commented on, showed deformation of the attached sheep red cells by microvilli from the R.F.C.

Such differences between mouse T and B lymphocytes as demonstrated by Matter et al. (1972) and in human/

human (albeit pathological) lymphocytes (Zucker-Franklin et al., 1974) might be useful in identifying T cells in mixed mononuclear populations.

6. Ultrastructure of monocytes and their interaction with red cells

i) The monocyte

The blood monocyte commonly possesses a horseshow shaped nucleus with finely dispersed chromatin and nucleoli are often present. The cytoplasm is voluminous and contains scattered small granules, which tend to be homogeneous and most of which are clustered at the nuclear indentation. The other cytoplasmic organelles include numerous cisternae of rough endoplasmic reticulum and a large active Golgi complex. Thus blood monocytes differ from other blood leukocytes in their profusion of cytoplasmic organelles, associated with active secretory granule formation. The granules in the monocyte are smaller, and fewer in number, than those of the polymorphonuclear leukocyte (Nichols & Bainton, 1975).

ii) Interaction of monocytes with red cells

Such interactions have recently been the subject of many reports (LoBuglio et al., 1967; /

1967; Abramson et al., 1970; Douglas and Huber, 1972). The original observation, that red cells sensitised with anti-D formed rosettes in vitro with human peripheral blood leukocytes, was made by Jandl and Tomlinson (1958), the identity of the leukocyte concerned, a monocyte, was established by Archer (1965).

The recent reports have focused on the regions of contact of red and white cell, the actual R.F.C. itself did not differ from the description given above.

In the majority of such rosettes the monocytes grasped the antibody-coated red cell by a variety of projections, either long or broad, the latter grasping the red cell over extensive areas.

The red cells in most instances underwent considerable distortion and deformity, in the region of the attachments. Often, there was complex interdigitation between short villous processes from the monocyte with indentations in the erythrocyte membrane, quite frequently, processes from the monocyte invaginated deeply into the attached red cell.

The closest distance between the monocyte and erythrocyte membrane was about 40 nm. Sometimes such areas of intimate contact were spaced at regular intervals.

iii)/

iii) Comparison of monocyte and lymphocyte interaction with erythrocytes

In a comparison of monocyte and lymphocyte interaction with sensitised red cells, the following differences were noted (Douglas and Huber, 1972). In monocytic interaction with anti-D sensitised cells, cytoplasmic processes extended towards the erythrocytes. Attachment sites, where they occurred, were not less than 40 nm. apart. Characteristically, deformation of the attached red cell was noted with monocytes. In the lymphocytic reaction with complement coated erythrocytes, the white and red cell membranes were between 10 and 20 nm. apart and contact often extended for several microns along the cell's diameter. Deformation of attached red cells was uncommon in this type of rosette.

Thus, in addition to having a different ultra-structure, monocytes and lymphocytes when forming rosettes differ markedly in their attitude to the attached red cell.

7. P.H.A. Transformation of lymphocytes as observed by transmission electron microscopy

In the previous chapter, homologous rosettes which appear following culture of lymphocytes with phytohaemagglutinin/

phytohaemagglutinin were described. Similar observations have been reported by other workers (Sheldon and Holborow, 1975a).

It is felt that a brief description of the ultrastructural features of P.H.A. stimulated cells is appropriate.

Characteristic changes in lymphocyte shape in mixed lymphocyte culture have been recognised for some years (McFarland and Heilman, 1965; McFarland et al., 1966; McFarland, 1969). Such changes include the formation of a uropod which imparts to the lymphocyte a 'hand mirror' appearance. Microspikes are often seen on the uropod and are not present on the rest of the lymphocyte membrane. The uropods contain cytoplasmic organelles such as microtubules, mitochondria, endoplasmic reticulum and pinocytotic vesicles. Lymphocytes appear to interact with other cells via this uropod, but actual cytoplasmic connection between such interacting cells has not been demonstrated (McFarland and Schechter, 1970).

A similar appearance has been noted in human lymphocytes stimulated by P.H.A. (Biberfeld, 1971a). Up to 20% of the cells developed uropods after 48 hours. Such uropods consistently contained the Golgi apparatus, the centrioles and associated microtubules./

microtubules. Other changes noted in P.H.A. stimulated cultures, are the development of long processes often with a bulbous tip (Clarke et al., 1971) and the ability to demonstrate light chain determinants, by immunoferritin techniques, on the surface of such cells (Biberfeld et al., 1971).

The multivesicular bodies which are seen in P.H.A. cultured cells, appear to be formed by a process of internalisation of part of the plasma membrane, to make small and large endocytotic vesicles (Biberfeld, 1971b). Two groups of workers have shown that P.H.A. stimulation results in cells with a paucity of endoplasmic reticulum, which contrasts with the prominent development of ribosomes, mitochondria, Golgi apparatus and nucleoli (Biberfeld, 1971a; Janossy et al., 1973). These workers also noted the development of cytoplasmic fibrils, close to the nuclear membrane.

8. The ultrastructure of the K-cell

The K-cell is considered to be responsible for the cytotoxic effect of human blood lymphocytes on antibody-coated chicken erythrocytes (Perlmann and Holm, 1969). It is conceivable that such/

such cells could be involved in autologous rosette formation in patients with auto-immune haemolytic anaemia. This especially, following the demonstration of such cytotoxicity in human systems (Dickmeiss, 1973). A brief review of their nature and ultrastructure will now be presented.

K-cells lack T cell markers and further, are distinct from mature B cells (Forman and Möller, 1973; Lärsson et al., 1975).

Their cytotoxic effect is expressed, only if the antibody on the target red cell has an intact Fc fragment (MacLennan, 1972; Perlmann et al., 1972).

Morphological observations by electron microscopy indicate that the small lymphocyte is the most likely candidate as effector cell in such systems (Biberfeld and Perlmann, 1970). In cultures, hosts of nucleated chicken erythrocytes and swollen, rounded up, chicken erythrocytes with eccentric, displaced nuclei were frequent. These deformed red cells were not necessarily in contact with lymphocytes. Two other features were noted, 0.5% of the lymphocytic cells phagocytosed chicken erythrocytes, and deformation of the erythrocyte surface in contact with lymphocytes was/

was seen with only minimal contact. These latter features have not been described in other publications reviewed in this section, and may well be unique for K cells.

9. Scanning Electron Microscopy

It has been claimed that the scanning electron microscope examination of lymphocytes enables differentiation between T and B lymphocytes. Thus, in one series, sheep cell rosetting cells (T) had a smooth, slightly irregular surface, and complement rosetting lymphocytes (B) had a villous surface (Polliack et al., 1974). It was noted in this series, that following rosette formation, a proportion of the R.F.C. had a more complex villous surface. This finding suggested that cell surface changes could be induced by the rosetting process.

It was further observed that SRBC were most frequently attached by circumscribed, pointlike areas, whereas in EAC rosettes, broader zones of contact between red and white cell were seen.

Similar findings were noted by Lin et al. (1973), who stated in addition, that B-lymphocytes often appeared to have processes deeply invaginating the tightly associated red cell and often/

*Complement rosettes

often a large portion of a lymphocyte microvillus was wrapped around the erythrocyte.

Another observation has been that of Lin and Wallach (1974), who found that cells cultured in Roswell Park Memorial Institute (R.P.M.I.) culture medium and rosetted with SRBC, developed short microvilli in the region of red cell contact and with increasing time these microvilli became long, undulating and branched. No distortion of the SRBC was noted. Some doubt on the significance of this observation, is raised by Jerrells and Hinrichs (1975) finding, that E rosettes* possessed a much more complex surface when prepared in R.P.M.I., than in Hanks Balanced Salt Solution (H.B.S.S.). This observation suggests that the human T cell is sensitive to medium changes and that in an enriched medium, such as R.P.M.I., has a morphology that may indicate a more physiologically active cell. The fact that cells in H.B.S.S. formed rosettes efficiently, indicates that the microvilli are not essential for erythrocyte adherence.

10. Design and reason for present investigation

The preceding review shows that though extensive knowledge of the ultrastructure of immune/

*sheep cell rosettes

immune R.F.C. exists, there is lack of similar information about non-immune R.F.C.. Evidence has been presented in the previous chapter, supporting the contention that the homologous and autologous R.F.C. in auto-immune haemolytic anaemia, are non-immune in nature. In an attempt to elaborate this idea, further studies, of an ultrastructural nature, were carried out, the results of which are now presented.

In addition the work provides new information on ultrastructural features of homologous R.F.C. in glandular fever, and from cultures undergoing stimulation with P.H.A.. The comparison of such R.F.C., which are on present evidence activated T cells (reviewed in the previous chapter), should help in more precise identification of those in A.I.H.A.. Such studies permit discussion of the application of ultrastructure to the identification of human activated T lymphocytes.

Finally evaluation of the use of scanning electron microscopy in the study of these forms of rosette is reported.

RESULTS

Plan

The detailed findings are presented in five sections:

- I Ultrastructural Features of R.F.C. common to all patients with Auto-Immune Haemolytic Anaemia (A.I.H.A.).
- II Features of R.F.C. which were unique or restricted to one or two patients with A.I.H.A..
- III Ultrastructural features of rosettes associated with glandular fever and P.H.A. stimulation.
- IV Ferritin labelling studies.
- V Scanning electron microscopy

Section I

A total of 172 rosette forming cells (R.F.C.) were identified and classified from ten patients with Auto-Immune Haemolytic Anaemia. Their main features are presented in the table.

CELL TYPE	NUMBER	TYPE OF CONTACT	DEFORMATION OF TARGET
LYMPHOCYTE	144	POINT	NO
MONOCYTE	21	POINT	YES
POLYMORPH	6	BROAD	NO
PLASMA CELL	1	BROAD	NO
TOTAL	172	-	-

Table A summary of cell types encountered in the total of 172 rosette forming cells. In addition, the type of contact with the red cell and the presence or absence of consequent deformation is given.

A. Overall pattern of rosetting reactions

Lymphocytes and monocytes were the two types of cell which commonly formed rosettes. Polymorphonuclear leucocytes were encountered in most preparations (usually less than 5%), but only six of all such cells were R.F.C., five of these were found in one autologous preparation (A.G.). No differences were found in the intrinsic ultrastructural appearance of the homologous or autologous lymphocytic R.F.C., though in a minority, to be described, differences were noted in the modes of binding by the target cell. Monocytes as R.F.C. were found more frequently in autologous preparations (15 from the total of 21).

B. Ultrastructure of lymphocyte rosettes

i) Size

Two typical rosetting lymphocytes are illustrated/

illustrated (Figs. I, II).

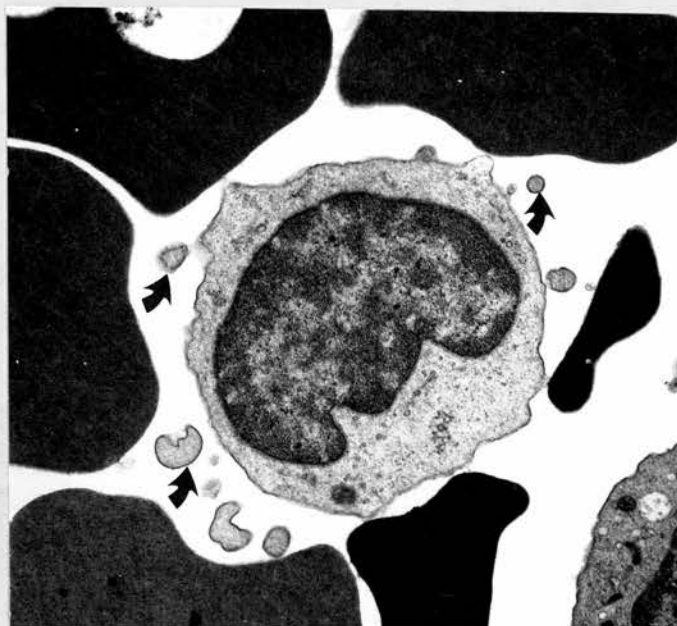


FIGURE I X 5000 Example of Homologous Rosetting Lymphocyte showing typical intrinsic ultrastructure and also the isolated cellular fragments (arrowed).

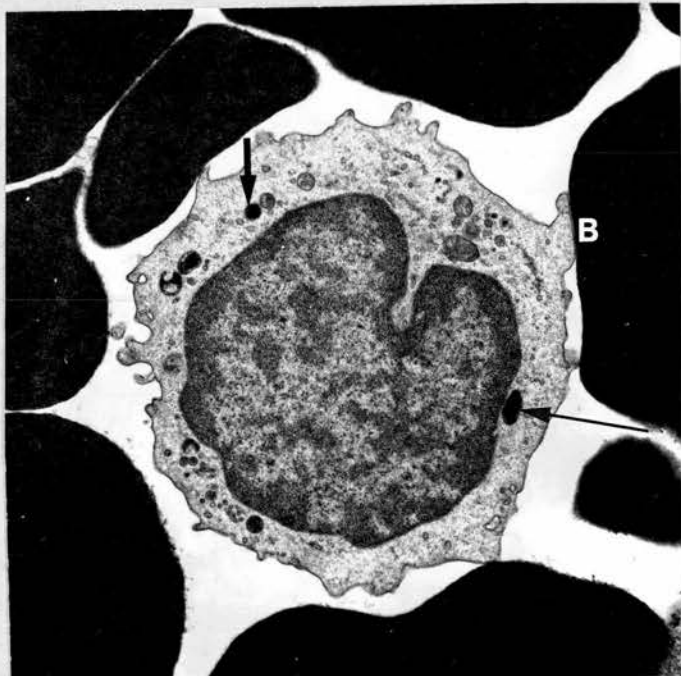


FIGURE II X 8000 Example of Homologous Rosetting Lymphocyte containing dense bodies (arrowed). One area of broad contact (B) is shown.

Such cells varied in size (4μ - 6μ). Small lymphocytes were more electron dense than the large lymphocytes. The nuclear cytoplasmic ratio in both types of cell was high.

ii) Nucleus

The nucleus was round in the majority of cases, but the outline was interrupted by indentations of varying depths. The heterochromatin was peripherally and centrally condensed. The euchromatin was finely dispersed. Half of such cells had a nucleolus and in some it was prominent (Fig. III).

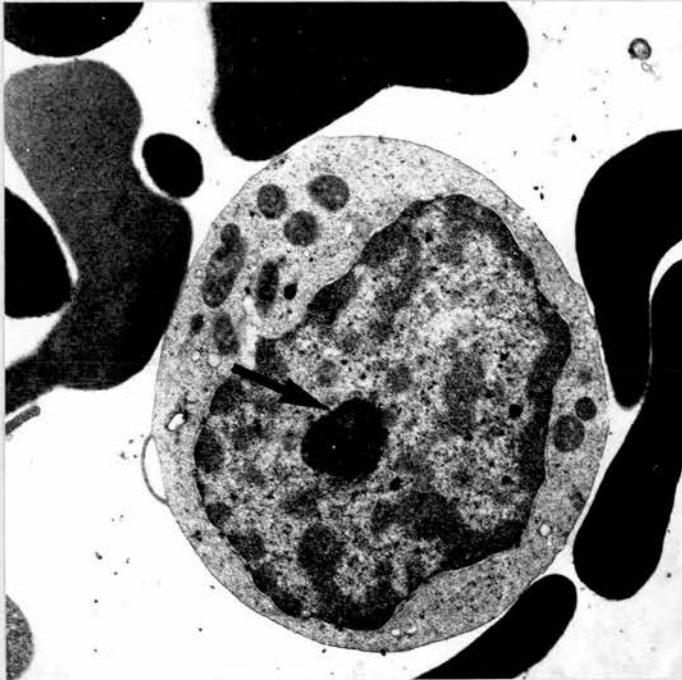


FIGURE III X 8000 Example of homologous rosetting cell with prominent nucleolus (arrowed).

iii) Cytoplasmic organelles

Ribosomes were frequent, most often monoribosomal, but cells with polyribosomes in a rosette pattern were frequently encountered. The ribosomes varied in electron density and occasionally were organised into profiles of rough endoplasmic reticulum. Other organelles were less frequently seen. They included large or medium sized mitochondria and a Golgi apparatus which was poorly developed. Occasionally a centriole was seen on section, as were dense bodies and multivesicular bodies. Two features worth special mention, were the presence of perinuclear microfilaments and secondly an area adjacent to the plasma membrane which was free of ribosomes (Fig. IV, V). This area was occupied by a fine granular matrix. These features were identified in 63 lymphocytic R.F.C.

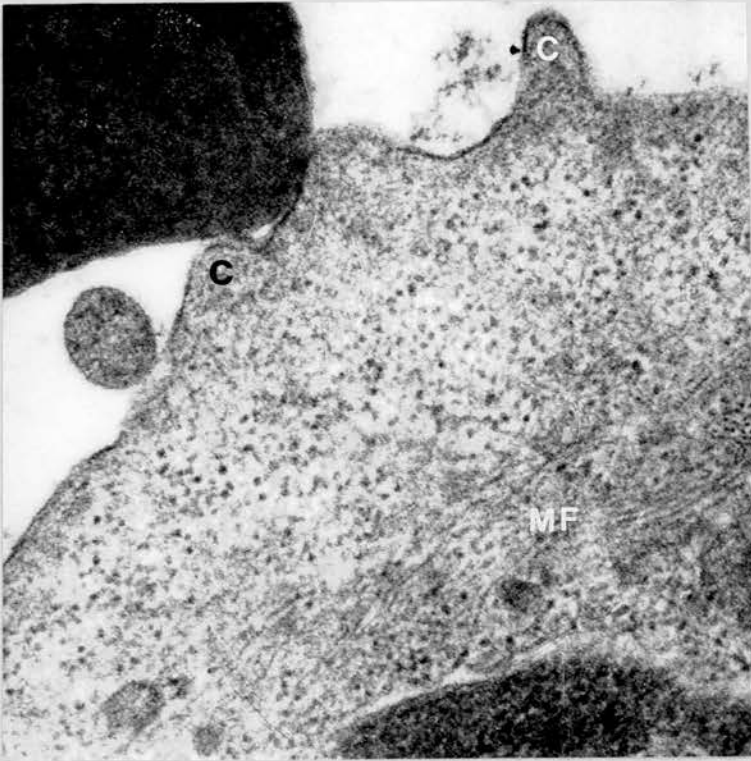


FIGURE IV X 80,000 Illustration of microfilaments (M.F.) and area of clearing under plasma membrane (C).

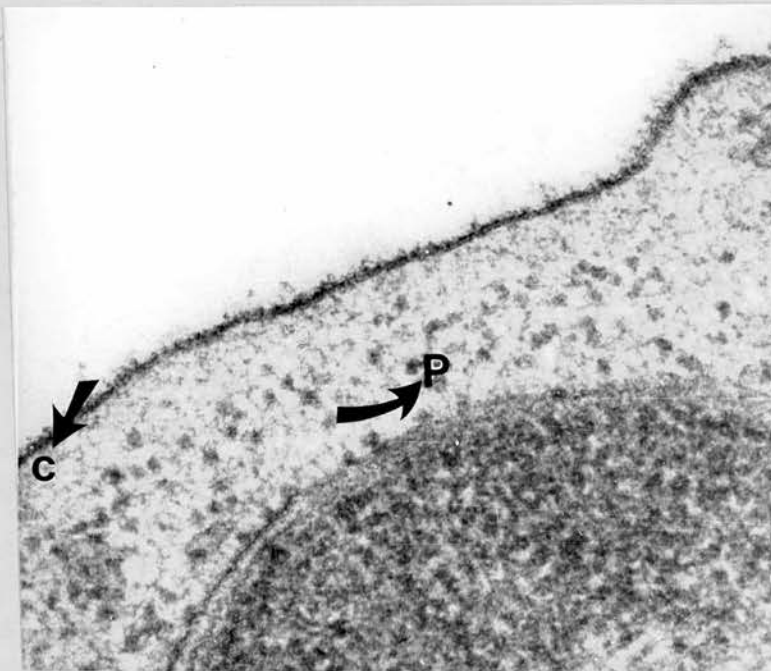


FIGURE V X 120,000 Clearing under plasma membrane (C) with the presence of occasional polyribosomes (P)

iv) Cytoplasmic Processes

The plasma membrane of most lymphocytes had a ruffled appearance, with short and long cytoplasmic villous processes. Surrounding many lymphocytes, but isolated from them, were circular cellular fragments (Fig. I). In some cases, serial sectioning showed them to be in continuity with the adjacent white cell, though such sectioning was not possible in all cases. The frequency and size of villous processes varied from cell to cell within individual patients and also between patients.

v) Type of binding with red cell

The most commonly encountered mode of contact was a pointlike or slightly extended membrane - membrane apposition. (Fig. VI).

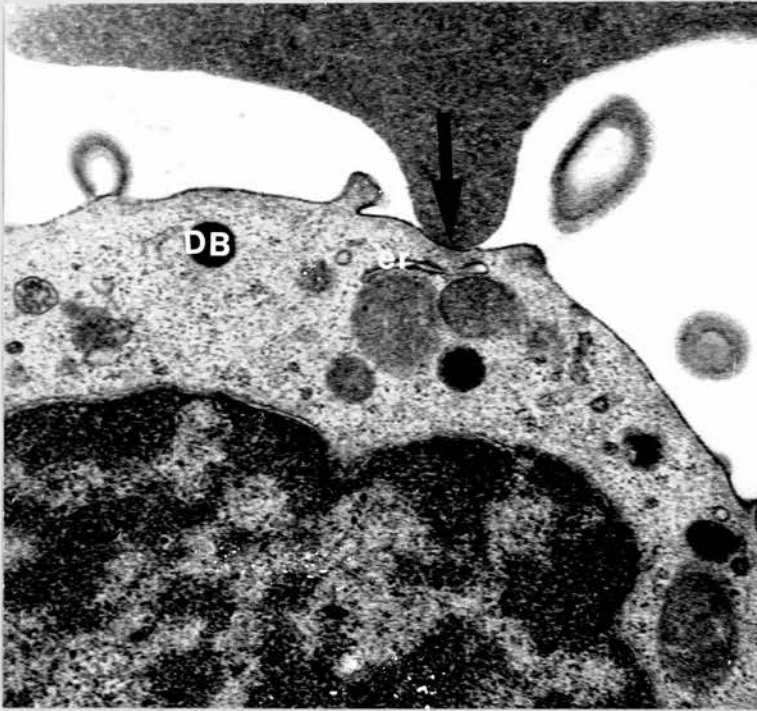


FIGURE VI X 30,000 Illustration of area of contact of white cell and erythrocyte (arrowed). Also shown are dense bodies (D.B.) and a short profile of endoplasmic reticulum (E.R.).

In a proportion of these, there was increased electron density of the white cell and erythrocyte membrane and adjacent cytoplasm (Fig. VII).

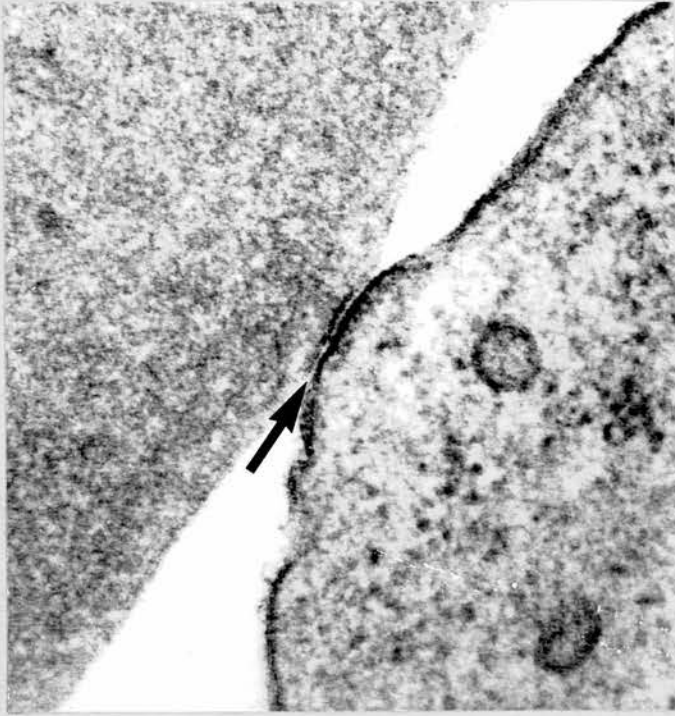


FIGURE VII X 120,000 Increased electron density of apposed red and white cell membranes (arrowed).

Often adhesion took place through three or four limited red or white cell processes (Fig. VIII, IX) or through longer processes, whose contact with the relevant cell could be ascertained, only by serial sectioning. Broad areas of membrane conjunction were seen in only four patients with any frequency and will be described in the second section.

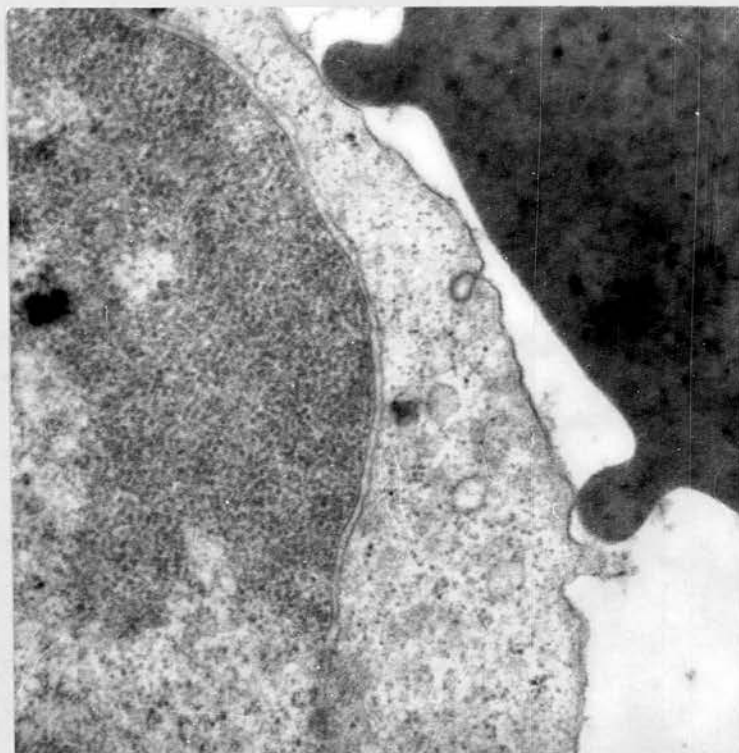


FIGURE VIII X 50,000 Example of adhesion via red cell processes.

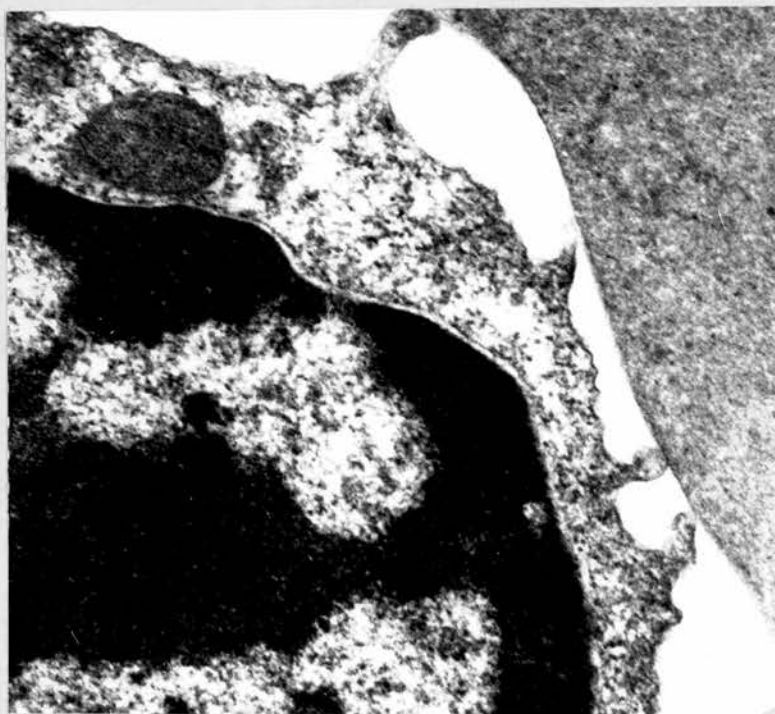


FIGURE IX X 50,000 Example of adhesion via white cell processes.

vi) Type of white cell-erythrocyte interaction

Deformation or invasion of the attached red cell did not take place to any significant degree in such rosettes. An example of the limited invasion sometimes encountered is shown (Fig. X).

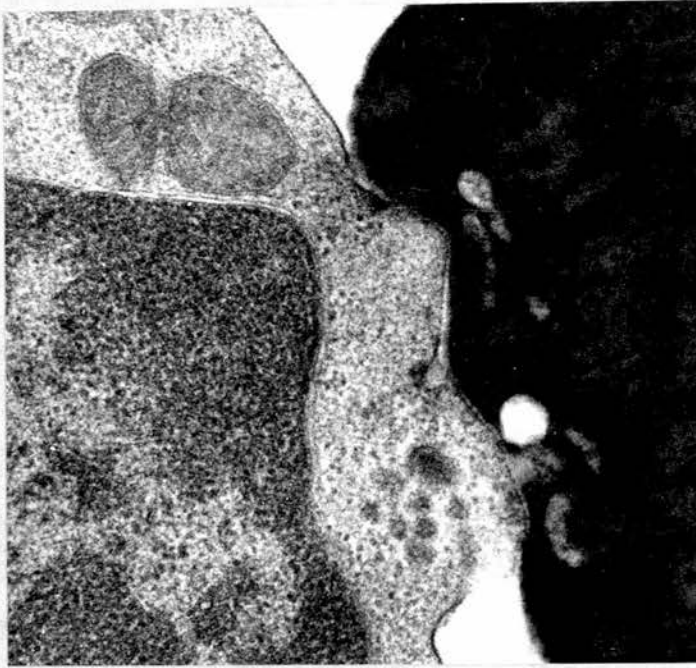


FIGURE X X 50,000 Ultrastructure of limited invasion of erythrocyte substance by a lymphocyte.

vii) Conclusions

Most lymphocytic R.F.C. were mature, with limited numbers of cytoplasmic organelles, apart from ribosomes. Often encountered, were microfilamentous structures and clearing of cytoplasmic organelles from an area immediately adjacent/

adjacent to the plasma membrane. The mode of attachment of the red cell was in most cases pointlike and deformation or invasion of the red cell was uncommon.

C. Ultrastructure of Monocyte Rosettes

i) Size

The monocytic R.F.C. was larger than the lymphocyte, varying in size from 7μ to 9μ .

(Fig. XI).

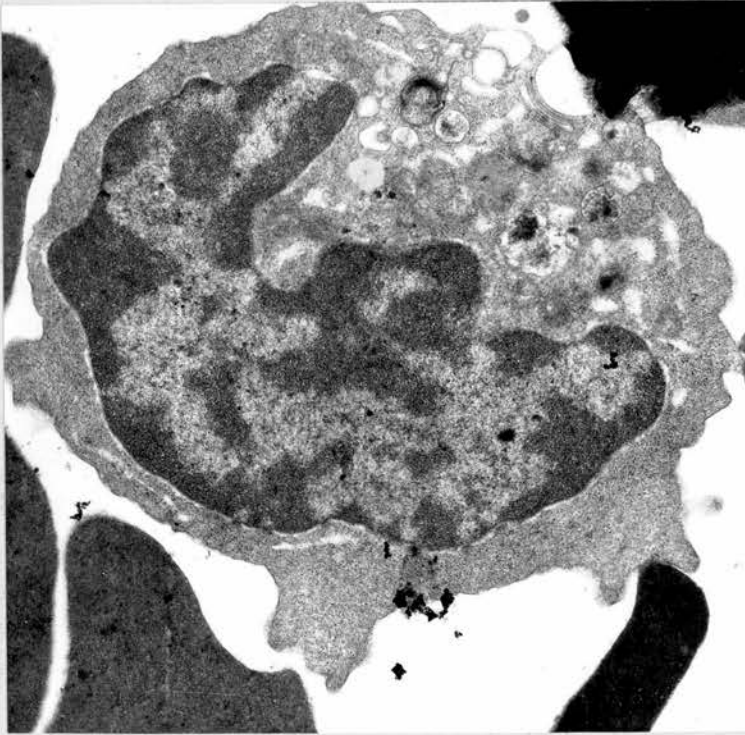


FIGURE XI X 12,000 Typical example of monocytic-rosette forming cell, showing the increased number of cytoplasmic organelles.

ii)/

ii) Nucleus

The nucleus was irregular with deep indentations. There was a thick layer of condensed heterochromatin adjacent to the nuclear membrane. Nucleoli were not often seen.

iii) Cytoplasmic organelles

Many more organelles were identified in this type of cell. There was a distinct tendency for them to aggregate in the convexity of the nucleus. Mitochondria were small. Numerous vacuoles containing electron dense and electron lucent material were present. The Golgi apparatus was well developed in most cases. The ribosomes were usually monoribosomal, occasionally short profiles of endoplasmic reticulum were seen terminating in, or in close apposition to mitochondria.

iv) Cytoplasmic processes

The cytoplasmic processes of this cell were more numerous and longer than in the lymphocytic R.F.C. The cells in general had a much more irregular outline.

v) Type of binding with red cell

There was equal division between broad and point contact by this type of cell.

vi)/

vi) Type of white cell-erythrocyte interaction

More aggressive interaction was seen with typical monocytes than with lymphocytes (Fig. XII, XIII).

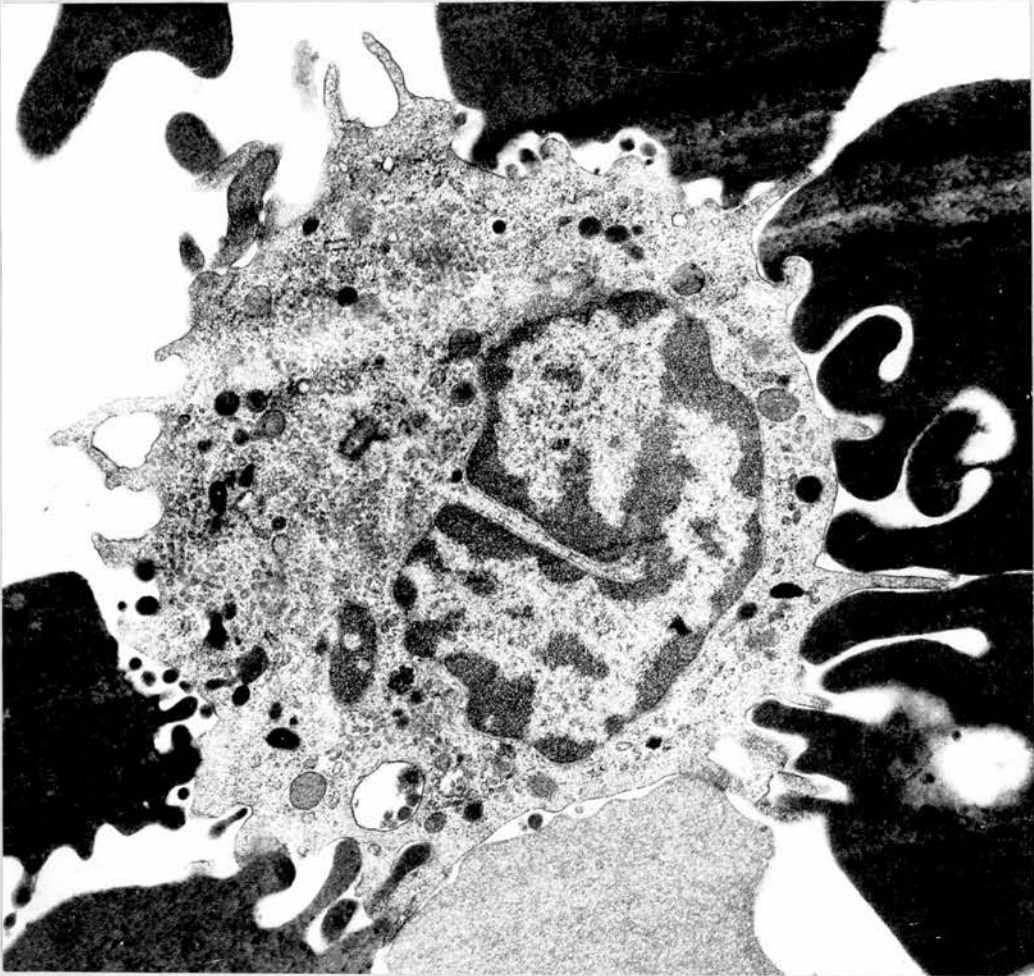


FIGURE XII X 15,000 Ultrastructure of aggressive interaction of monocyte with attached red cells.

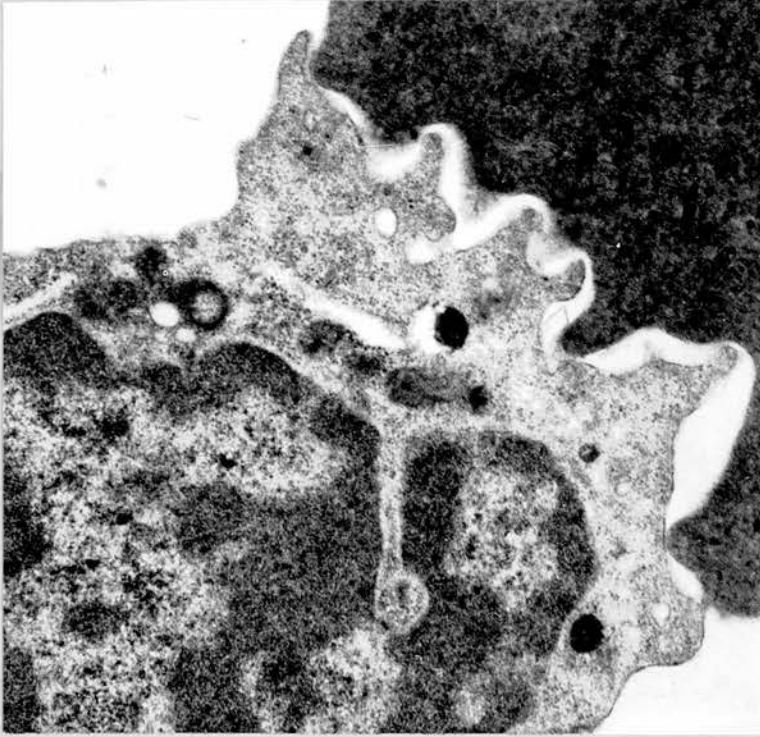


FIGURE XIII X 30,000 A higher power view of deformation of erythrocyte caused by monocytic-rosette forming cell.

The red cells in contact were usually deformed but not consistently. In addition, red cells in close relationship but not in actual contact in the plane of section, often contained fragments of white cell tissue. (Fig. XIV).

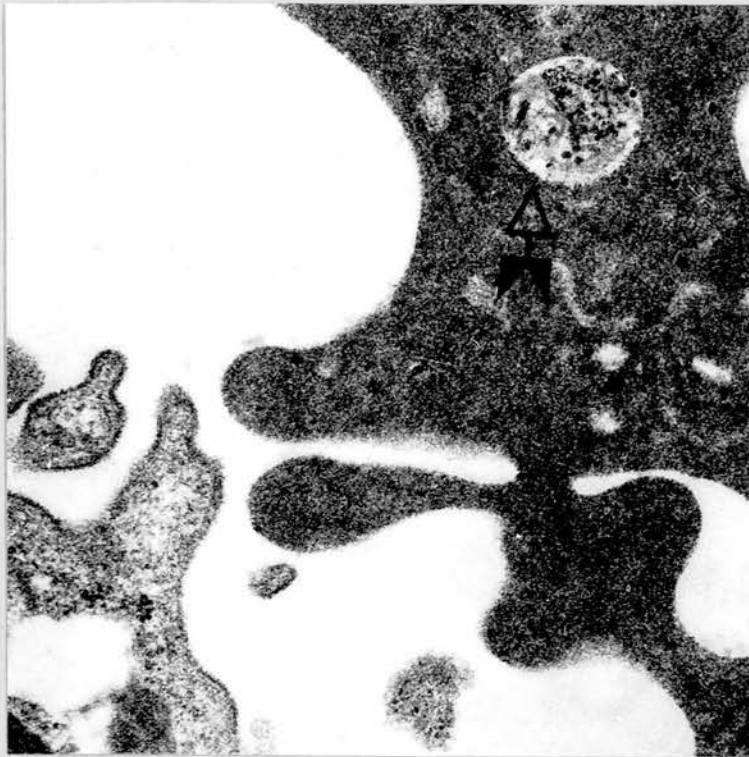


FIGURE XIV X 50,000 An example of invasion of an erythrocyte by a monocytic cellular process (arrowed).

Further, red cells in close proximity, with no evidence of white cell contact, often showed gross deformation (Fig. XV).



FIGURE XV X 20,000 A deformed red cell, not having direct contact with a leukocyte but in close proximity to a monocyte.

The ingestion of erythrocytes by such cells was often accompanied by a smoother membranous outline and a quiescent appearance (Fig. XVI).

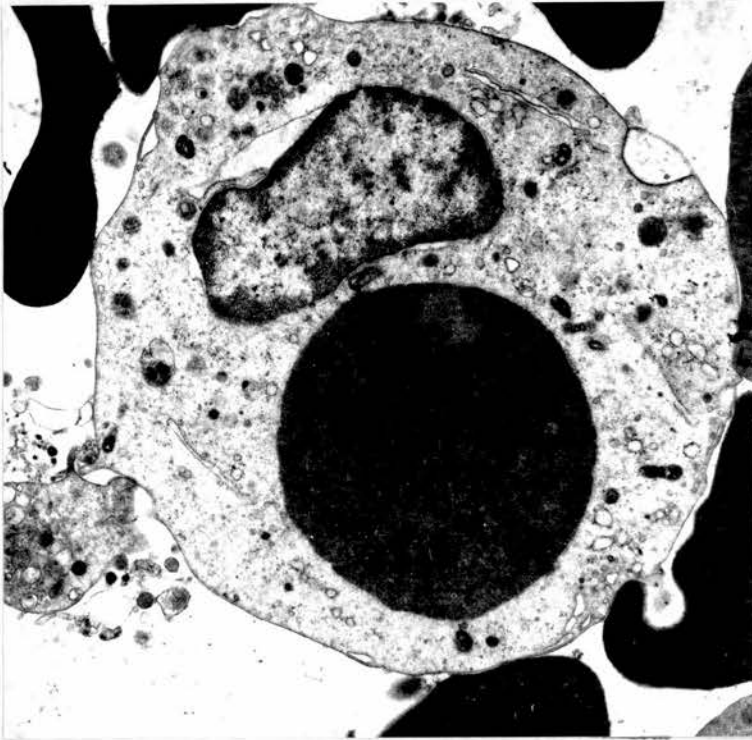


FIGURE XVI X 12,000 Illustration of the quiescent appearance sometimes produced following red cell ingestion.

vii) Conclusions

All monocytic R.F.C. were mature and were distinguished by their irregular cytoplasmic and nuclear outline, and the increased number of cytoplasmic organelles. The most characteristic feature was the marked red cell deformation they produced.

Section II

A. Individual Variations in Rosetting Reactions

i) Broad Areas of Contact

Extensive areas of adhesion were seen in four patients, one had cold agglutinin disease and the features in this patient will be described later. The three other patients with broad areas of contact were: M.B., autologous rosettes; A.W., autologous rosettes; J.K., autologous and homologous rosettes. In the illustration of the R.F.C. (Fig. XVII) from J.K., broad contact areas are predominant.

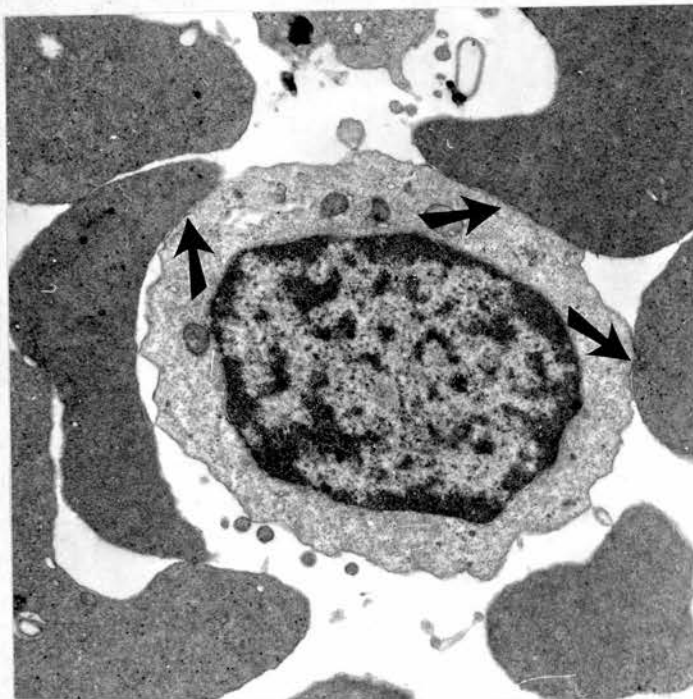


FIGURE XVII X 12,000 An example of a homologous rosette in which most of the contact areas are broad (arrowed).

An area of broad contact is shown at high power (Fig. XVIII).



FIGURE XVIII X 80,000 A high power view of broad contact. Arrowed are irregularly spaced points of closer apposition and increased membrane density.

Electron dense bridges are seen at irregular intervals along the length of apposition. The intermembranous gap is 20 nm. at these intervals.

ii)/

ii) Comparison of R.F.C. from blood, spleen and lymph node

Such a comparison was possible in M.P., a 78 year old patient who underwent splenectomy. At the time of laparotomy a lymph node was also removed.

(a) Blood

In this patient the lymphocytic R.F.C. showed more variation in ultrastructure than that seen in others. Variation encountered was decreasing nuclear-cytoplasmic ratios, concomitant increase in cytoplasmic organelles and irregularity of nuclear outline.

(b) Spleen

The variation in cell type observed in the blood was not noted in the spleen. A lymphocytic-R.F.C. is illustrated (Fig. XIX).

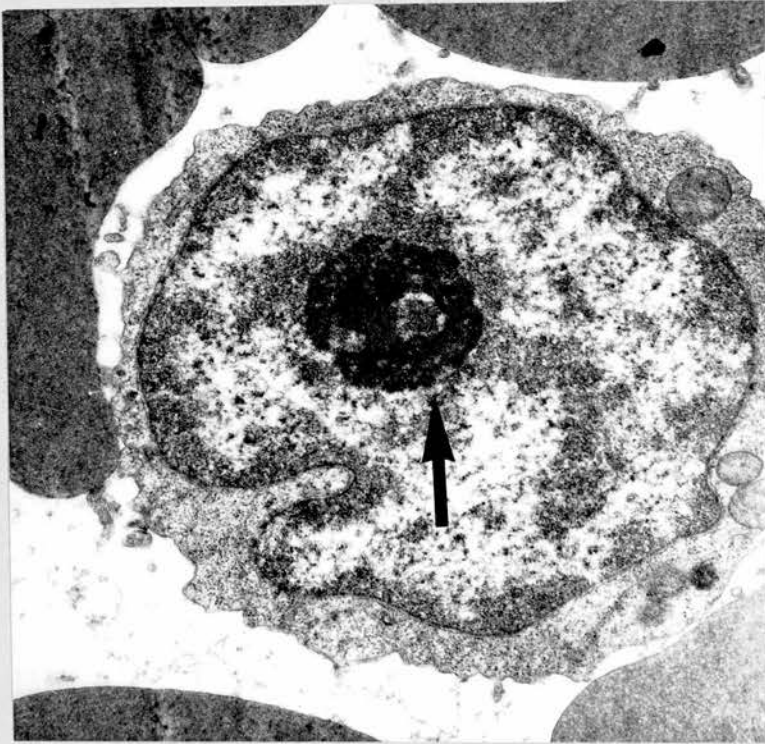


FIGURE XIX X 20,000 A splenic lymphocytic rosette forming cell. The prominent nucleolus is arrowed.

A/

A higher power microphotograph shows a long cytoplasmic process making contact with a red cell in a homologous rosette (Fig. XX).

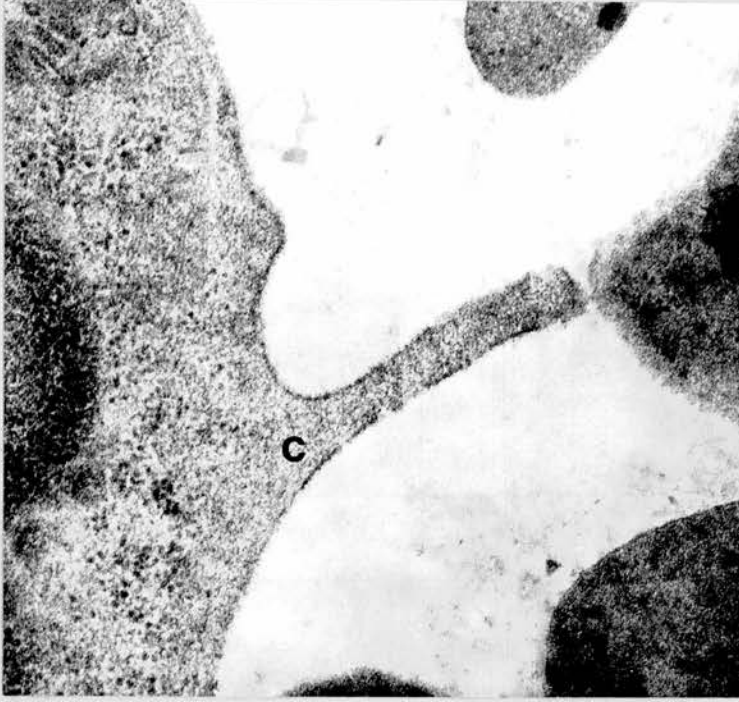


FIGURE XX X 30,000 Cellular process making contact with erythrocyte in splenic lymphocytic rosette forming cell. Clearing under the plasma membrane is well shown (C).

The fine matrix under the plasma membrane is well demonstrated here. In this organ monocytic R.F.C. made up to 20% of the total rosetting population.

(c) Lymph Node

The homologous and autologous rosettes identified in the node did not have any distinguishing features from the general lymphocytic R.F.C. described in section I.

iii)/

iii) High monocytic contribution

In three patients, T.K., A.G., W.M., there was a higher contribution of monocytes to the autologous R.F.C.

iv) Uropod Formation

T.K. and A.G. were the only patients in whom lymphocytes with distinct uropods were identified. They are illustrated (Fig. XXI), the cell shows the formation of microspikes.



FIGURE XXI X 12,000 An example of a lymphocyte with uropod and microspikes (M.S.)

v) Cold agglutinin disease

In this patient, A.P., monocytes formed 10% of the R.F.C. and the red cell deformation caused/

caused by such cells is illustrated (Fig. XXII).

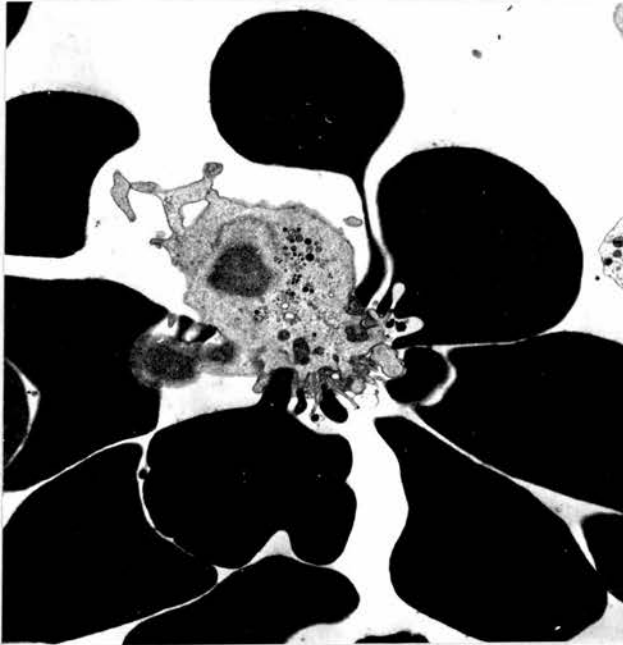


FIGURE XXII X 5,000 Section through one pole of a monocyte from a patient with cold agglutinin disease. Red cell deformation is clearly seen.

There was in addition similar but much less marked deformation by lymphocytic cells (Fig. XXIII).

The one illustrated is unusual because of the large numbers of clear vacuoles it contains.

The predominant mode of adhesion was broad.

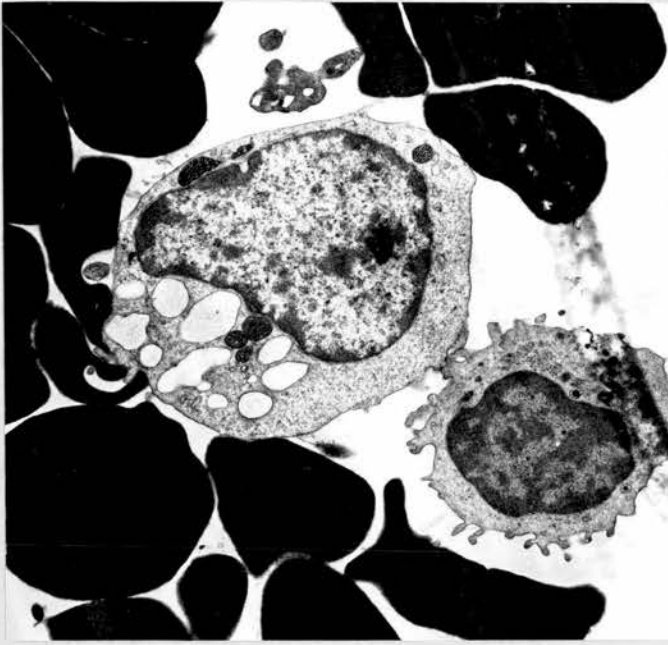


FIGURE XXIII X 5,000 Lymphocyte-rosette forming cell from patient with cold agglutinin disease.

vi) Increased number of attached red cells

M.G. differed from all other patients because the lymphocytic R.F.C. were often composed of up to nine red cells attached to the central cell (Fig. XXIV). In other subjects, rosettes usually consisted of three, and at the most five red cells attached to the R.F.C.

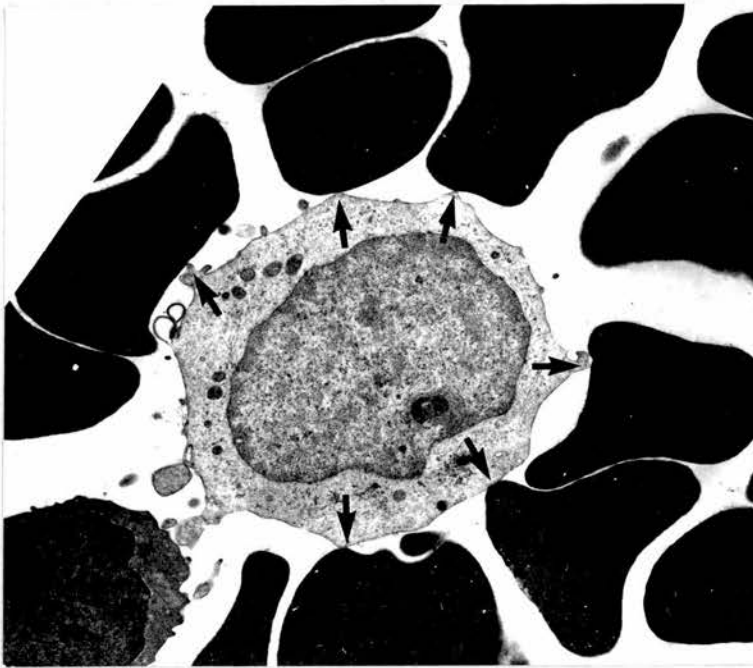


FIGURE XXIV X 5,000 Example of lymphocyte-
rosette forming cell from patient M.G. There is
definite connection with six red blood cells
(arrowed).

vii) Cells difficult to classify

In nine R.F.C. the central cell was more
difficult than usual to classify as lymphocyte or
monocyte. The final illustration from this section
is an example (Fig. XXV).

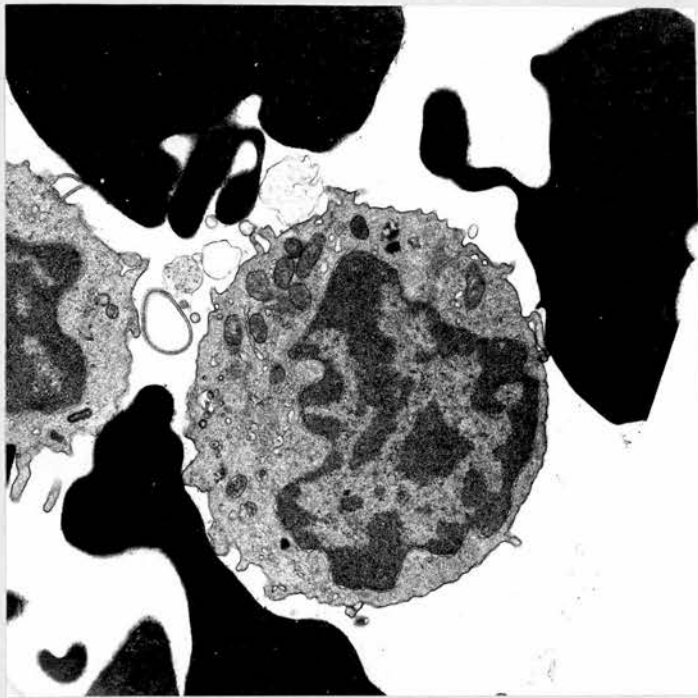


FIGURE XXV X 8,000 Example of rosette forming cell which presented some difficulty of classification. Finally classified as Lymphocytic rosette forming cell.

It is round, with a slightly ruffled outline and has numerous large mitochondria. Present are electron lucent vesicles but there are few profiles of endoplasmic reticulum. The ribosomes are monoribosomal. The nucleus is irregular with abundant heterochromatin.

Section III

A. Glandular Fever

All rosettes identified were lymphocytes, but differed from lymphocytic R.F.C. found in A.I.H.A. in several ways.

i) Nucleus

The nuclear cytoplasmic ratio was higher in most cells than those in A.I.H.A. (Fig. XXVI).

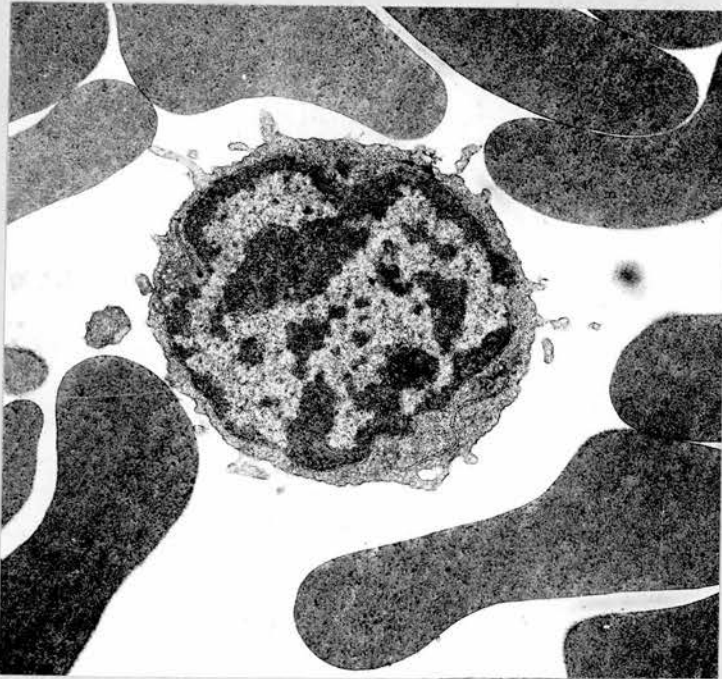


FIGURE XXVI X 8,000 Ultrastructural features of homologous rosette from patient with glandular fever.

ii) Cytoplasmic Processes

These were longer and more numerous than in A.I.H.A. Direct contact between the R.F.C. and/

and the red cell was not seen in many preparations but small isolated fragments of white cell were present in the intervening space (Fig. XXVII).

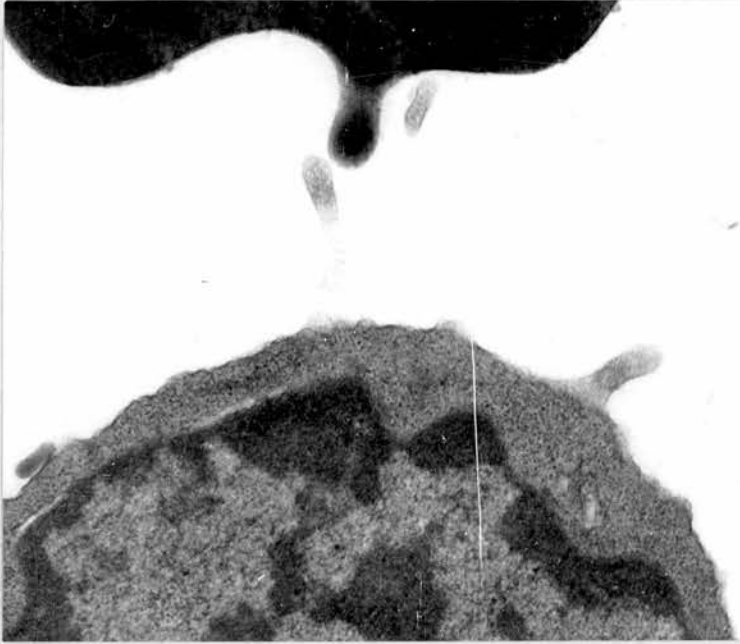


FIGURE XXVII X 30,000 High power view of cytoplasmic processes in glandular fever rosette forming cell.

iii) Type of binding with red cell

This was pointlike in all cases. The continuity, between the R.F.C. and red cell, had to be confirmed on many more occasions than in A.I.H.A. by serial sectioning.

iv) Type of white cell erythrocyte interaction

No deformation of attached red cells was seen.

B./

B. Phytohaemagglutinin Stimulated Rosettes

All the R.F.C. were lymphocytes (Fig. XXVIII), but were much larger than those either in A.I.H.A. or glandular fever. The features distinguishing them from A.I.H.A. lymphocytic R.F.C. are now described.

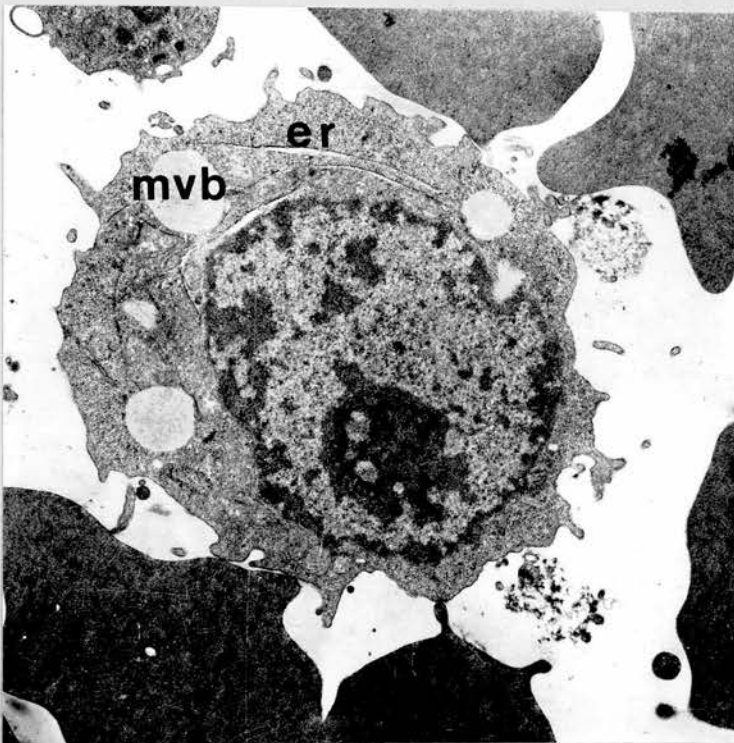


FIGURE XXVIII X 12,000 Ultrastructure of homologous rosette found in P.H.A. stimulated cultures. Shown are multivesicular bodies (MVB), and long strands of endoplasmic reticulum (ER).

i) Nucleus

There was scant heterochromatin and prominent single or double nucleoli.

ii)/

ii) Organelles

The presence of very long profiles of endoplasmic reticulum and large variably electron dense multivesicular bodies characterised these cells. In addition increased numbers of mono- and poly-ribosomes were present.

iii) Cytoplasmic Processes

Many more cytoplasmic processes extended from these cells than in A.I.H.A. and were more variable in thickness.

iv) Type of binding with red cell

Like A.I.H.A. attachment was mainly pointlike, but equally contributed to by red or white cell processes (Fig. XXIX).

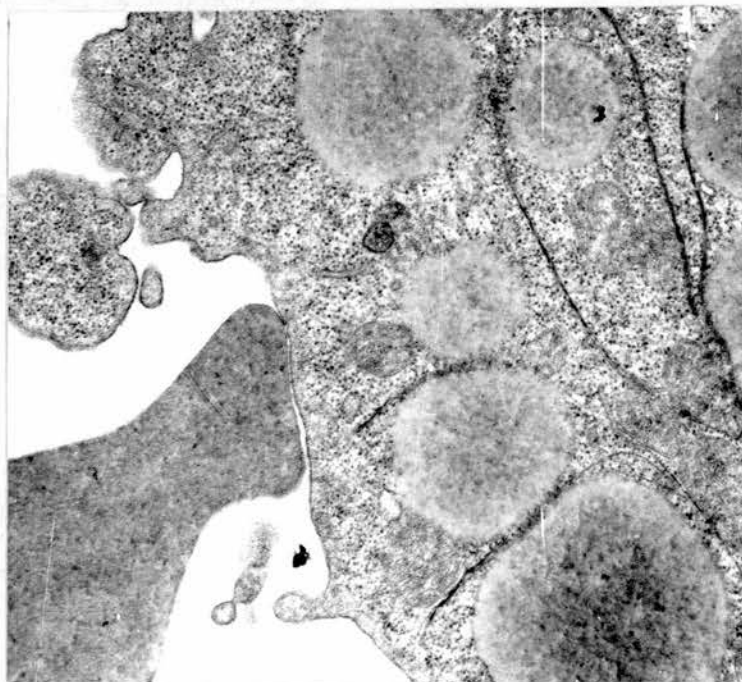


FIGURE XXIX X 30,000 Area of contact in P.H.A. induced homologous rosette forming cell.

v) Type of white cell - erythrocyte interaction

No deformation or invasion was seen.

Feature common to PHA cultures and Glandular Fever

In both cases a ribosome free area was visualised under the plasma membrane. This was occupied by an amorphous matrix. This feature, seen and remarked on in A.I.H.A. lymphocytic R.F.C., was more prominent in both these conditions.

Section IVFerritin Studies

The total number of rosettes encountered in these preparations was small, eight in all, from five patients with A.I.H.A. The significant feature seen in all such rosettes was the absence of ferritin from the membrane, both at low power and also in high powered examination of junctional areas (Fig. XXX).

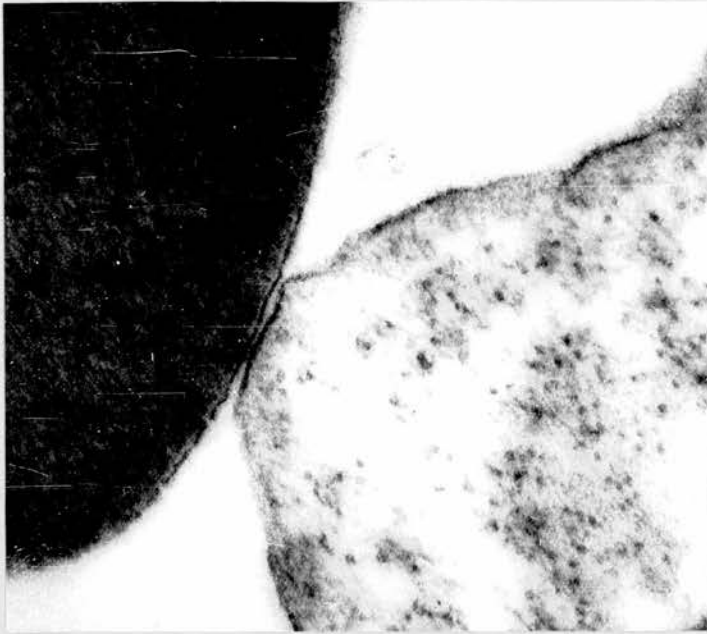


FIGURE XXX X 80,000 Absence of ferritin molecules at a point of contact in ferritin preparation.

Present in all preparations were a small number of cells which showed membrane ferritin. (Fig. XXXI).

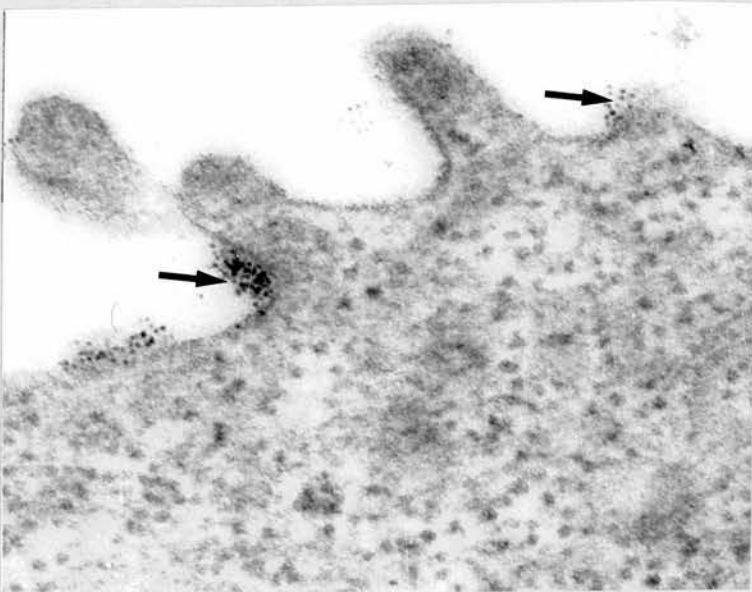


FIGURE XXXI X 120,000 Appearance produced by membrane associated ferritin in positive cells.

No cells with such membrane bound ferritin were identified as rosettes.

Section V Scanning Electron Microscopy

i) Homologous Rosette, A.I.H.A.

(a) Rosette Forming Cell

Seventeen such cells were examined from four patients. All were spherical. Ten of the seventeen presented the surface appearance illustrated in Fig. XXXII with a few limited, blunt villous processes.

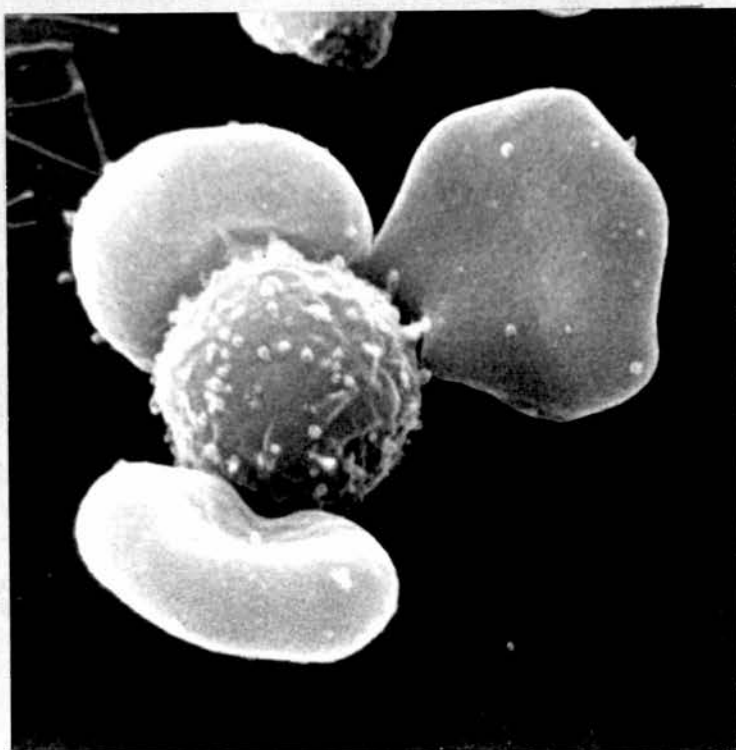


FIGURE XXXII X 14,200 Scanning electron microscopy of homologous rosette forming cell from patient with A.I.H.A. Clearly shown is the surface topography and mode of contact.

Five had more complex villous processes (Fig. XXXIII) and the other two a surface which presented an intermediate appearance between these two (Fig. XXXIV)

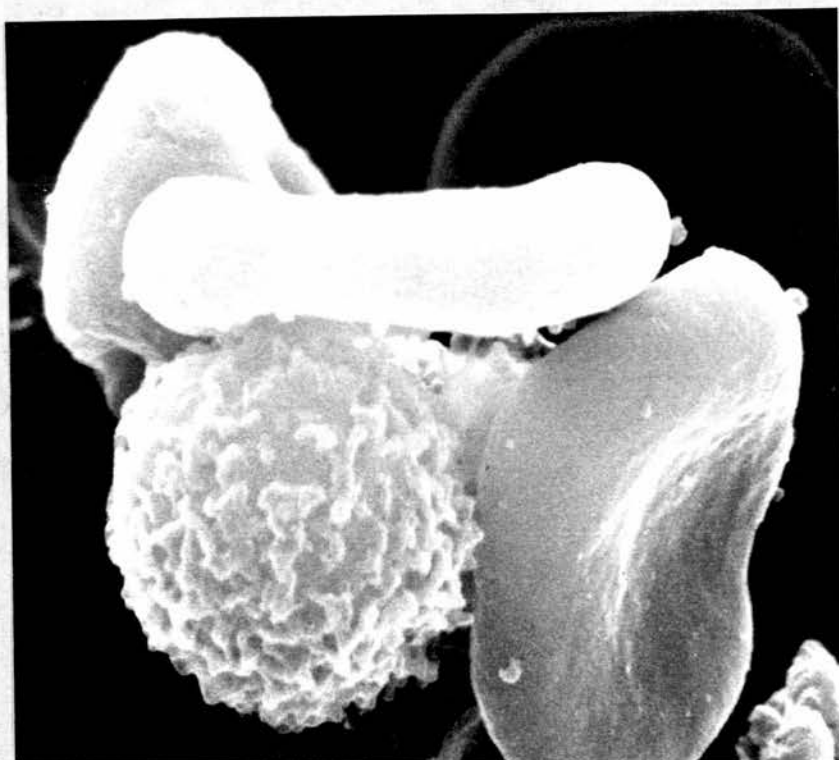


FIGURE XXXIII X 21,200 A second example of a homologous rosette forming cell from A.I.H.A. The surface is more complex.

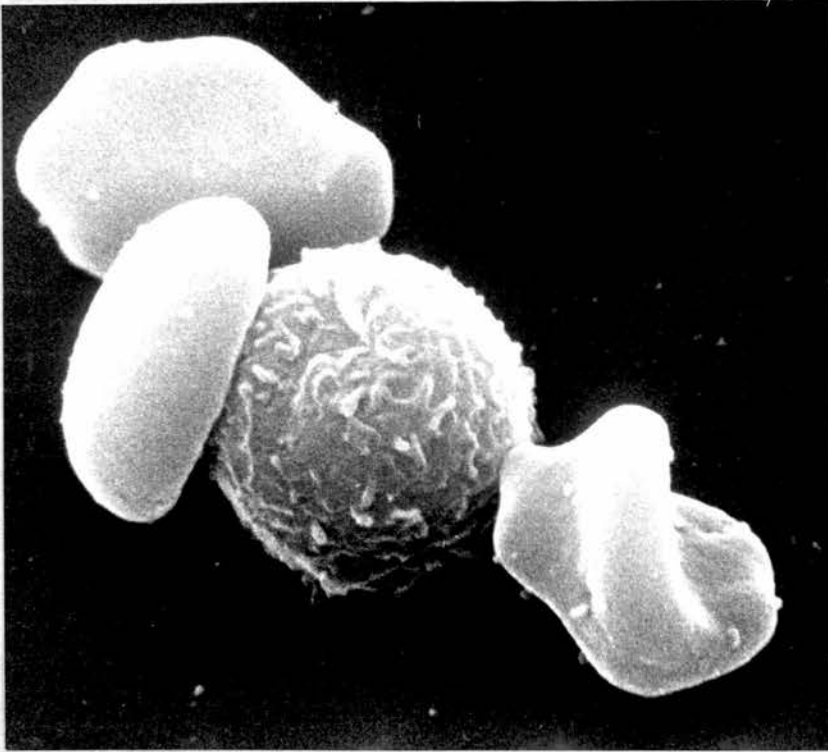


FIGURE XXXIV X 18,600 Third form of A.I.H.A. homologous rosette forming cell encountered in scanning electron microscopic preparations.

(b) Types of Binding with Red Cell

These were in most cases circumscribed pointlike areas due to blunt villous processes from either the R.F.C. (Fig. XXXV) or attached erythrocyte (Fig. XXXVI). Occasionally the processes were longer (Fig. XXXVII) and sometimes a combination of processes from both red and white cell was encountered (Fig. XXXVIII)

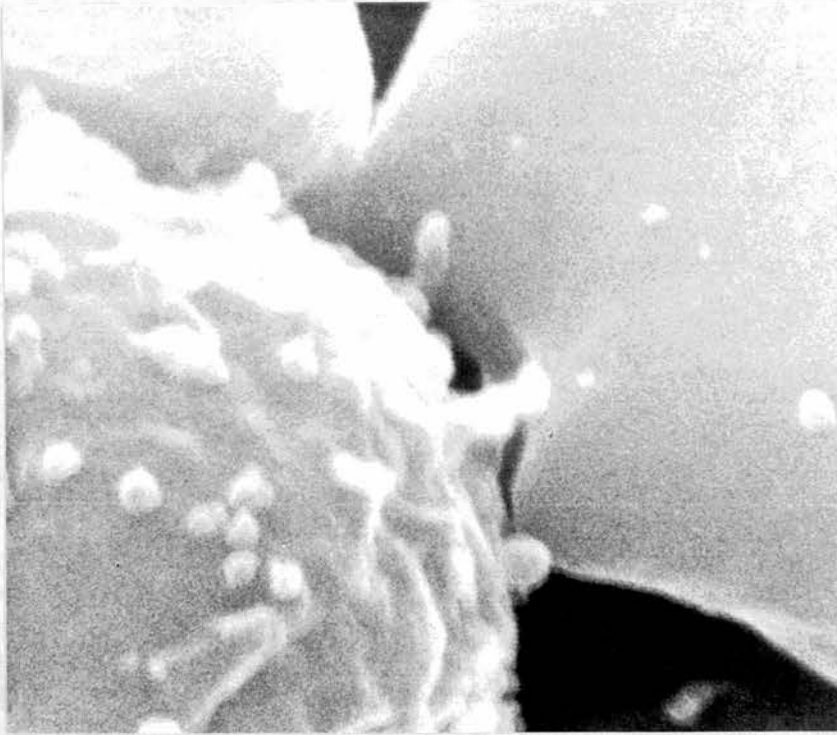


FIGURE XXXV X 42,600 High powered view of contact of rosetting cell and erythrocyte. These features correspond with those found in Fig. IX.

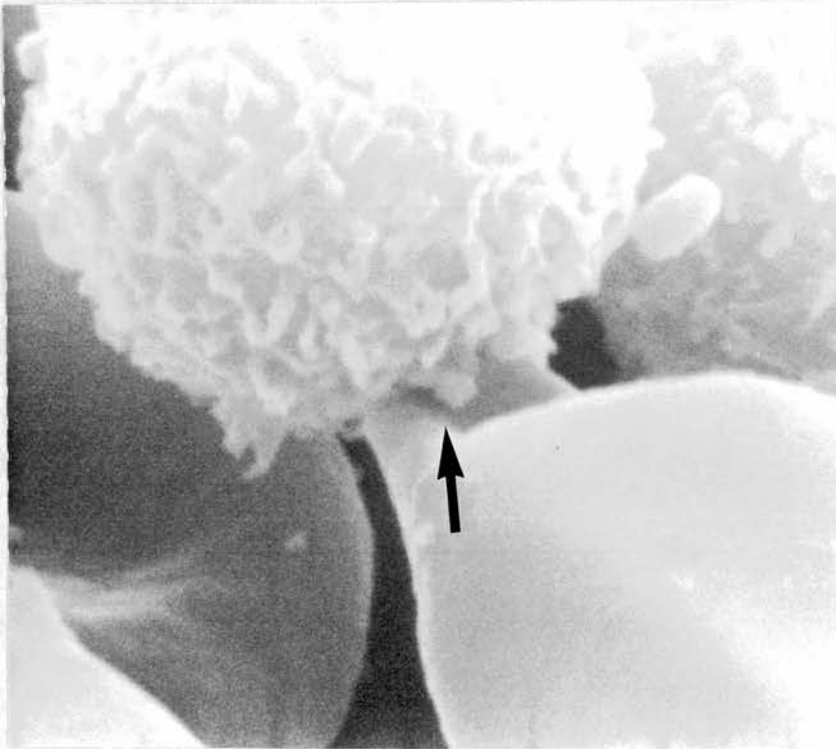


FIGURE XXXVI X 30,200 Erythrocyte process (arrowed) making contact with rosetting leukocyte.

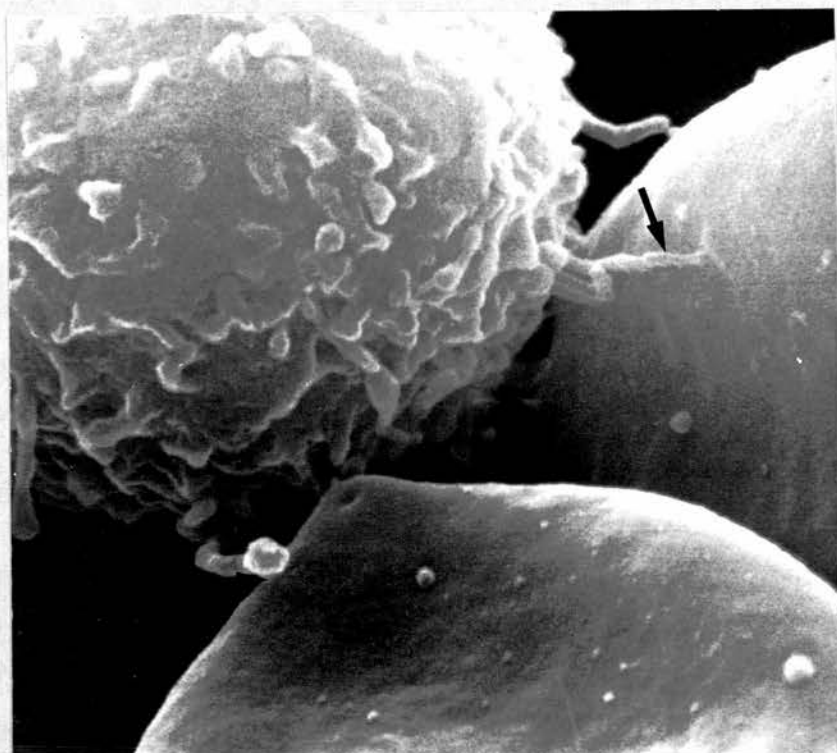


FIGURE XXXVII X 31,200 An example of more tenuous connection by thin cytoplasmic processes (arrowed).

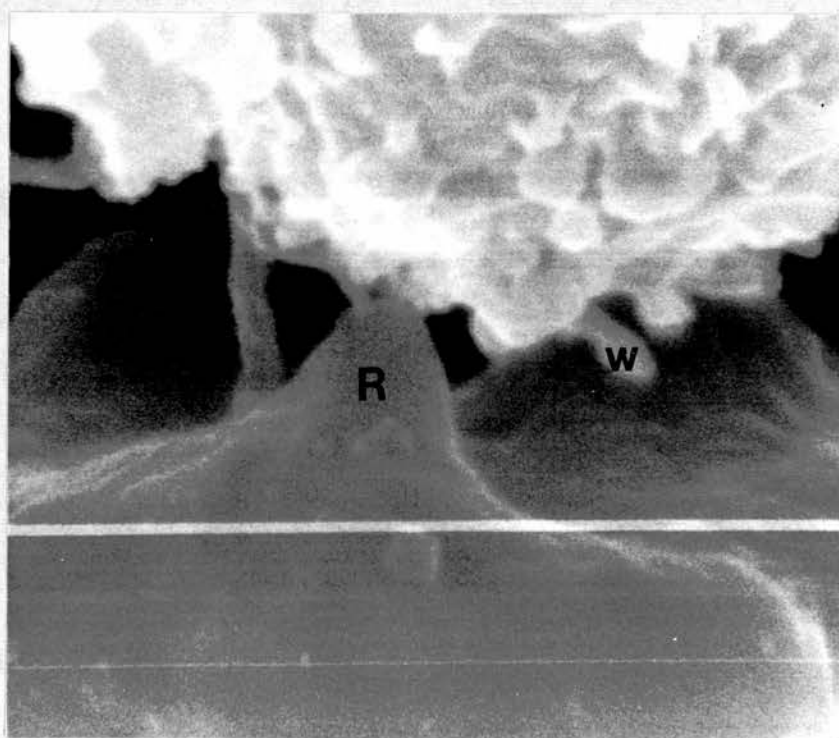


FIGURE XXXVIII X 51,000 Combination of red (R) and white (W) cell processes contributing to adhesion

In only one rosette was a broader interface between erythrocyte and R.F.C. identified (Fig. XXXIX). In this one illustration the intermembranous gap was 150 nm.

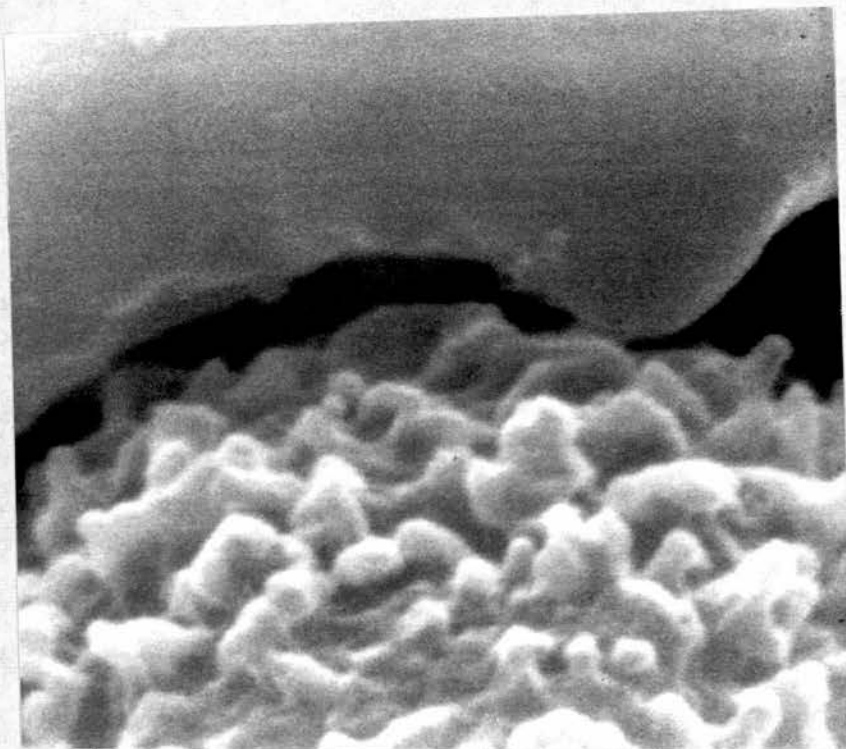


FIGURE XXXIX X 51,000 The only example encountered in scanning electron microscopy of broad contact.

No deformation of the attached red cells was seen and usually only three and at the most four red cells were attached.

ii) Sheep Rosetting Cells

(a) Rosette Forming Cell

These cells were spherical with a complex villous surface. The villi were mainly blunt/

blunt and short (Fig. XXXX).



FIGURE XXXX X 16,400 Sheep rosetting cell showing complex villous surface. More red cells are attached than in homologous rosettes.

(b) Type of binding with red cell

In contrast to the homologous R.F.C. in A.I.H.A., many more red cells were attached. Commonly at least four and up to ten. Again, attachment of erythrocyte to R.F.C. was by circumscribed pointlike areas. There was no deformation of attached red cells.

iii) Non-Rosetting Cells

In general non-rosetting cells could not be distinguished in terms of their surface topography/

topography from sheep cell rosetting cells. They were more villous than ten of the seventeen homologous R.F.C. encountered. Monocytes were identified by their characteristic folded membrane (Fig. XXXXI). None of these cells formed rosettes in these preparations.

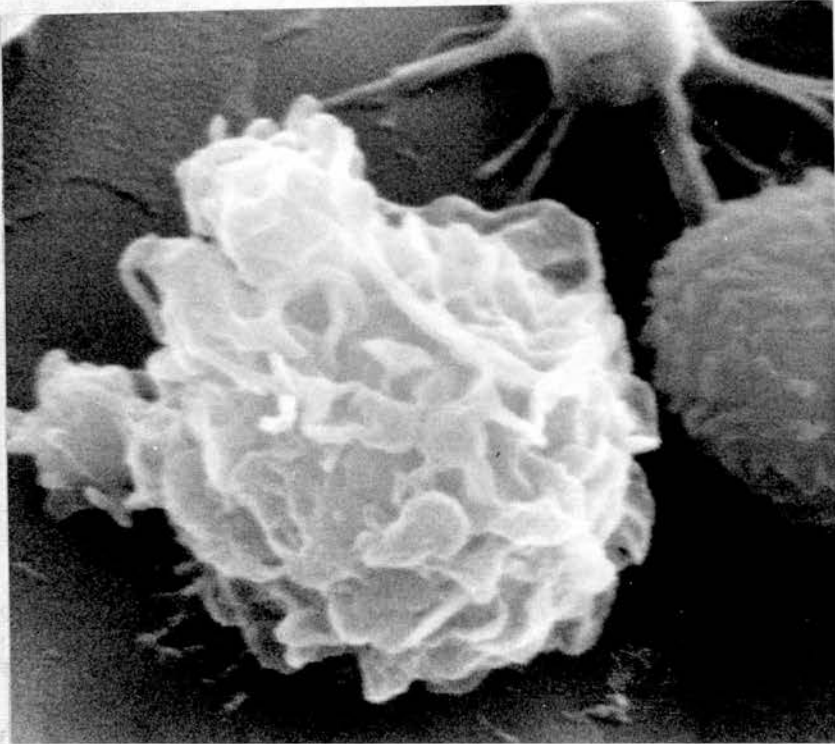


FIGURE XXXXI X 21,400 An example of a monocyte encountered on scanning electron microscopy.

iv) Other Conditions

Cells from glandular fever were not examined. An attempt was made with P.H.A. cultured cells. Though no rosettes were encountered the cells presented the appearance shown, with limited folds of membrane, the remainder of the cell being smooth. (Fig. XXXXII).

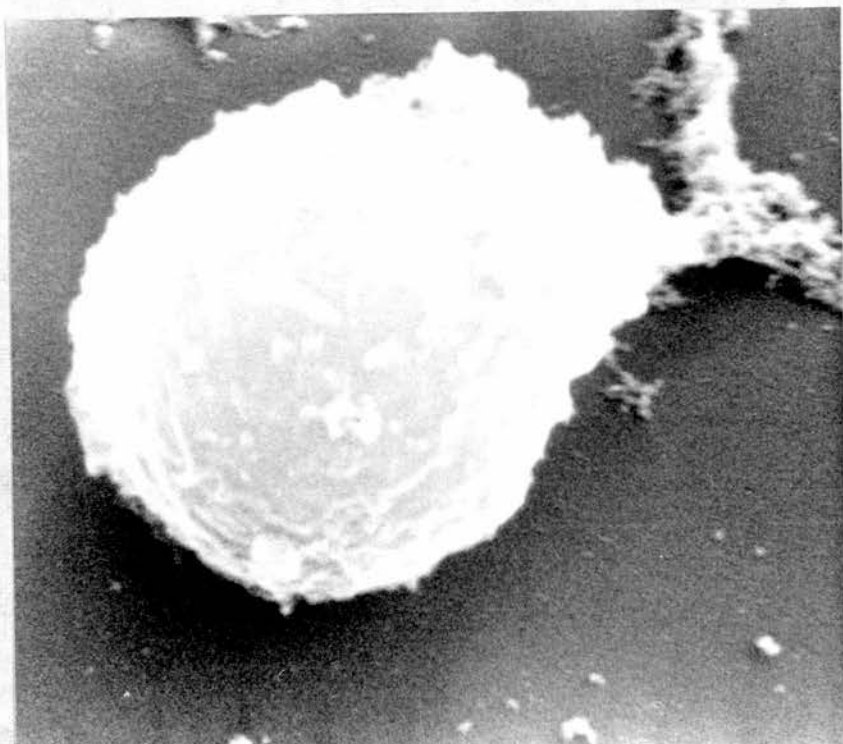


FIGURE XXXXII X 19.400 Lymphocyte from P.H.A. culture (72 h). The surface membrane of the cell is clearly shown.

DISCUSSION

1. Prelude
2. Are the lymphocytic R.F.C. Immune or Non-Immune?
3. Are the lymphocytic R.F.C. thymus or bone marrow derived?
4. Monocytic rosetting forming cells.
5. Polymorphonuclear R.F.C..
6. Plasma cell.
7. Areas of binding.
8. Comments on other features.
9. Scanning electron microscopy.

Summary.

DISCUSSION

1. Prelude

The ultrastructural studies of the rosette forming cell in auto-immune haemolytic anaemia, in glandular fever and in phytohaemagglutinin stimulated cell cultures were carried out to provide information on four major points.

Firstly to give an accurate estimate of the incidence of lymphocyte, monocyte and polymorphonuclear leucocyte contributing to the total R.F.C.. This has been achieved, and requires no further expansion, other than to say firstly, that some cells were encountered that were difficult to classify, either as lymphocyte or monocyte. This difficulty has been noted by other authors (Van Furth, 1970). The problem was however minor and only arose in the case of nine R.F.C., most of which were finally called lymphocytes. Secondly the ratio distribution of each cell type was not equal in each patient. Thus in three, and most prominently in autologous preparations, there was a high contribution of monocytes (up to 55%). An increased clinical severity of the disease resulting in heavier sensitisation of the autologous red cell might partially explain this finding. That/

That it is not the total explanation, is suggested by the lack of similar findings in patients with equivalent degrees of clinical activity.

The last three points were firstly, to determine whether or not the rosette identified was immune or non-immune. Secondly to see whether there was evidence enabling identification of the lymphocytic R.F.C. as of T or B derivation. Finally, it was hoped such a study would provide precise information of the modes of red and white cell interaction with different types of R.F.C. These three points will now be separately discussed.

2. Are the Lymphocytic R.F.C. Immune or Non-Immune?

As regards the intrinsic ultrastructure of the 143 lymphocytic R.F.C., they did not form a completely homogeneous population. Thus there was variation in size of the cells, in the number and development of mitochondria, in the presence or absence of nucleolus and in the amount of nuclear heterochromatin.

None of these cells however showed much development of endoplasmic reticulum or Golgi apparatus, and no evidence was found of transition to antibody producing (plasma) cells. Thus, as regards these latter points, these cells were homogeneous./

homogeneous. Most reports which have described immune rosettes (Storb et al., 1967; Storb et al., 1969; Duffus and Allan, 1971; Gudat et al., 1971; Gudat et al., 1970), have remarked on the heterogeneity of the lymphocyte which forms the immune rosetting population, in particular the development of endoplasmic reticulum and also on the contribution of plasma cells.

The two reports found in the literature concerning non-immune rosettes, one of unimmunised mice (Reyes and Bach, 1971) and the other of P.H.A. stimulated human lymphocytes (Levy et al., 1975), both suggest that such cells are homogeneous, particularly with regard to lack of development of endoplasmic reticulum.

Thus the lymphocytic R.F.C. described in this thesis correspond to the non-immune group of rosetting cells. It is important to establish this, as cells secreting auto-red cell antibody would not be unexpected in the peripheral blood in A.I.H.A. These cells would be likely to form rosettes due to specific immune mechanisms. Immune rosettes due to specific antibody production have been described in humans by Elson and Bradley (1970) and Feizi et al. (1973).

The/

The presence or absence of surface immunoglobulin, does not indicate whether a cell is cytologically equipped to elaborate antibody or not. For example, normal and neoplastic plasma cells lack surface immunoglobulin (Pernis et al., 1970; Paraskevas et al., 1970). As far as B lymphocytes and their progeny are concerned, increasing development of endoplasmic reticulum is associated with a reduction in surface immunoglobulin (Ishii et al., 1974; Zucker-Franklin and Berney, 1972). It follows, that B lymphocytes cannot be excluded by a separate demonstration, of either lack of surface immunoglobulin or of endoplasmic reticulum. For more definitive exclusion, absence of both features is desirable.

The absence of surface Ig on the homologous R.F.C. as demonstrated in the ferritin labelling experiments, together with their lack of development of cytoplasmic endoplasmic reticulum, is strong evidence that these R.F.C. do not carry, nor are they producing significant amounts of immunoglobulin. The conclusion that can be drawn from this, is that the R.F.C. are more likely to be non-immune, as immune R.F.C. require immunoglobulin on their surface/

surface to act as the medium for specific adhesion.

Further support for the non-immune nature of the rosette identified, was that in all but one patient (M.G.), the R.F.C. interacted with only three or four red cells. This argument has been used in the clinical section, but is valid here too. Thus rosette formation with B cells usually results in many more adherent erythrocytes than with T cells (Haskill et al., 1972).

In summary, the homologous R.F.C. and autologous R.F.C. are thought on the evidence presented here to be non-immune. This conclusion by itself does not mean that they are all thymus derived but there is no positive evidence of a high B cell contribution to the population.

3. Are the lymphocytic R.F.C. thymus or bone marrow derived?

The evidence discussed here is concerned with intrinsic ultrastructural features revealed by transmission electron microscopy. Definite microfilaments or clearing of the cytoplasm, immediately adjacent to the plasma membrane of organelles, was found in 63 of the lymphocytic R.F.C. This clearing was of varying depth and the area was occupied by a greyish amorphous matrix. These features have been described in thymus/

thymus derived cells in the mouse and increase on activation of the cell (Matter et al., 1972). Similar morphological studies of human non-immune rosettes, have confirmed the usefulness of such changes as indications of activation (Levy et al., 1975). In addition, perinuclear fibrils have been noted in lymphocytes stimulated with PHA and Con A* (Biberfeld, 1971a; Janossy et al., 1973).

Using the criteria described, 63 (44%) of the lymphocytic R.F.C. have been classified as T cells. If microfilaments are used as a sign of activation, then most of these R.F.C. are activated.

The evidence on purely morphological grounds for the nature of the remaining cells is less absolute, but taken in accordance with the other laboratory results, given in the previous chapter, especially those of the mixed rosettes, it would seem likely that a high proportion are thymus dependent.

The morphological features of the P.H.A. stimulated and glandular fever rosettes support this interpretation. Most workers accept that P.H.A. predominantly stimulates thymus derived cells (Greaves et al., 1974b). There is also evidence that the predominant proliferating cell/

*conconavalin-A.

cell in glandular fever is a T cell (Carter, 1975). The rosetting cells, encountered in both these sets of experiments, had microfilamentous structures, as revealed by an expanded grey amorphous area adjacent to the plasma membrane. This feature was more marked in both these experimental situations than in R.F.C. found in A.I.H.A.

The presence of these microfilamentous structures, in both glandular fever and P.H.A. stimulated R.F.C., is evidence that changes similar to those described in the mouse, occur following activation of T lymphocytes in man. They add further support to the contention, that 44% of the lymphocytic R.F.C. in A.I.H.A. which show similar features, are T cells.

4. Monocytic rosette forming cells

These were encountered most commonly in autologous preparations. Such an incidence is most likely explained by the presence on circulating monocytes of complement and immunoglobulin receptors (Huber and Fudenberg, 1970). Though such interaction was first described in 1965 (Archer), only recently has it been possible to interpret it in terms of cell membrane receptors. As red cells in most cases of A.I.H.A. are sensitised/

sensitised by IgG and complement they would be expected to adhere to monocytes via corresponding receptors, (but see argument in Chapter III, p.112.)

As such sensitisation is not present on homologous erythrocytes, the formation of homologous monocytic R.F.C. is less easily explained. Three possibilities are proffered. Firstly, such adherence could be due to the recognition by the monocyte of minor antigenic differences on the homologous erythrocyte or secondly, to binding of a proportion of the red cells because of membrane surface changes subsequent to ageing. The latter explanation seems least likely, as no increase in homologous R.F.C. was found on testing with increasingly old erythrocytes. A final explanation could be that monocytes from patients with A.I.H.A. are activated. Such a phenomenon has been described by Evans (1975) in in vitro experiments. Thus macrophage cultures can lyse target cells used to immunise the donor. After contact with the specific target cell, the macrophages are activated and are able to inhibit growth of target cells which are immunologically unrelated. This phenomenon is thought to be related to cytophilic antibody on the macrophage surface. In the case of the experiments described in this thesis, the cytophilic/

cytophilic antibody could be optimally deposited on monocytes at 37° and its subsequent elution prevented by the low temperature of the experiment (Berken and Benacerraf, 1966). Reyes and Bach (1971) were unable to explain the formation of monocytic R.F.C. in unimmunised mice.

5. Polymorphonuclear R.F.C.

Seven of the eight polymorphonuclear R.F.C. were found in the autologous preparation from one patient (A.G.). In this patient the red cells were sensitised with complement. As C3 receptors have been described in polymorphonuclear leukocytes (Lay and Nussenzweig, 1968), this might well have been the mode of interaction in this case.

6. Plasma Cell

The single plasma cell rosette was in fact the only typical plasma cell encountered in the whole investigation. Its significance, primarily because of its low frequency, obviously cannot be evaluated.

7. Areas of Binding

Fundamental differences between the two main types of R.F.C. and their interaction with red/
red/

red cells was identified. In this section information derived from transmission electron microscopy will be discussed.

(i) Lymphocytes

The predominant mode of contact was pointlike. This finding was common for R.F.C. in A.I.H.A., glandular fever and in PHA stimulated cultures. The attachments took the form of white or red cell processes, the former being most frequent. As serial sectioning was often required to demonstrate the continuity of the white cell process and its adhesion to the red cell, this suggested that such processes were tendrils, rather than prolonged folds of the plasma membrane. That such a conclusion was correct, in many cases, was shown by morphology revealed on scanning electron microscopy which will be discussed later.

Point-adherence has been described in many types of rosette, both immune and non-immune (Storb et al., 1969; Gudat and Villiger, 1973; Reyes and Bach, 1971). Many workers have however commented on the broader areas of contact which occur, when erythrocytes sensitised with complement or immunoglobulin, are used as the test particle to detect B lymphocytes (Bentwich et al., 1973; Chen et al., 1972; Douglas and Huber, 1972).

The/

The difference of this broader contact of B lymphocytes with sensitised erythrocytes, from that made by T lymphocytes with S.R.B.C., has been attributed to the relatively few sites of attachment available on the T cell. Douglas and Huber (1972) propose that this reflects a fundamental difference between plasma membranes of B and T cells.

Formation of point adherence would account for the known fragility of rosettes which are formed by T cells (Bach, 1973a; Hogg and Greaves, 1972). It would also fit with the known sparcity of binding sites on T cells as compared to B cells (Nossal et al., 1972; Haskill et al., 1972).

One variable makes comparison of different reports in the literature difficult, and that is temperature. Some experiments showing narrow zones of contact were performed at 4°C (Storb et al., 1967; Storb et al., 1969; Bentwich et al., 1973), those showing broad zones of contact have often been carried out at 37°C (Douglas and Huber, 1972; Elson et al., 1972; Zucker-Franklin and Berney, 1972). Thus temperature might be critical, in determining whether 'close fit' or point adherence is encountered. Temperature changes cause movement of surface immunoglobulin when it is linked by multivalent ligands (Taylor et al., 1971) and of other

other surface antigens (Roelants et al., 1973). Similar redistribution of surface molecules with temperature, if it applies to the surface receptors responsible for non-immune rosette formation, might well cause different patterns of adherence.

Increased electron density of the red and white cell membrane at points of contact has been noted previously (Gudat and Villiger, 1973). A possible explanation of this phenomenon may be afforded by the observation by Zucker-Franklin and Berney (1972). They found that strands, bridging lymphocytes and red cells, appeared to be continuous with structures in the cytoplasm of the white cell. Aggregation of such 'anchors' at points of contact might explain the increased density of membrane and cytoplasm.

Broad areas of contact by such cells were infrequent. In some cases they were limited, in others quite extensive. In the P.H.A. cultures, lengthening of contact areas reported by Levy et al. (1975) was not seen.

Irregularly spaced, electron dense bridges were seen extending between the membranes of such close contacts. Other workers have commented on such features at contact areas of R.F.C. (Gudat and Villiger, 1973; Zucker-Franklin and Berney, 1972). These authors suggested that they represented the/
the/

the receptors responsible for binding.

At such bridges there was often a more intimate apposition of the membranes. The exact nature of these bridges remains unclear, but it is tempting to suggest that they are the binding sites.

(ii) Monocytes

These cells differed from lymphocytes in their interaction with red cells in two ways. The points of adhesion were often broader but a major difference was the deformation of red cells attached to monocytes that occurred. In addition to deformation there was actual invasion, as shown by the presence of isolated white cell fragments within the red cell substance. Such aggressive interaction has been described before with antibody-coated red blood cells (Lobuglio et al., 1967; Abramson et al., 1970; Douglas and Huber, 1972).

Similar appearances were noted in the interaction of monocytes with homologous red cells, the latter being non-sensitised. Thus such aggressive interaction does not require a sensitised red cell.

8. Comments on other features

Though carefully looked for, the features which have to date been described as characterising the/
the/

the K-cell, such as its lymphocytic nature contrasting with invasive properties and also its ability to phagocytose (Biberfeld and Perlmann, 1970), were not found.

In one patient (M.P.), the lymphocytic R.F.C. showed a wider variation in ultrastructural features than that found in other patients. The reason for this is not clear. Similar variation was not observed in the spleen and lymph node R.F.C. of the same patient. This subject has not shown any characteristic clinical difference from the other patients.

As uropod formation is considered to be a sign of lymphocyte activation and more particularly that of T cells, in guinea pigs at least (Rosenstreich et al., 1972), it was surprising that so few patients demonstrated this feature. The explanation may be, that the cells examined corresponded to 'T2 cells' in the mouse, which are activated, but have not reached the stage of uropod formation (Matter et al., 1972).

The findings in the one patient studied with cold agglutinin disease, contrast with those of Feizi et al. (1973). They found that the dominant mode of adherence by lymphoid cells was pointlike. They did encounter close fit adherence but this was/

was with plasma cells. The reason for this difference is not clear, as the experimental technique used was virtually the same. In many of the rosettes studied in this thesis in cold agglutinin disease, the increased deformation of attached red cells indicated that the R.F.C. were of a different nature than those of warm antibody A.I.H.A..

9. Scanning Electron Microscopy

The results of scanning electron microscopy, although technically satisfactory, yielded a limited amount of information. They confirmed that point contact was the main form of adhesion between erythrocyte and lymphocyte. The surface topography of all the homologous R.F.C. was consistent with the features of T lymphocytes described in the literature.

The other finding which does require further evaluation, was that 10/17 of the homologous R.F.C. had surface features more akin to P.H.A. stimulated cells than the unstimulated T cell, the latter being identified by its SRBC-rosetting ability. Whether this is a valid comparison, which would add further to the evidence indicating that the homologous R.F.C. is an activated lymphocyte, requires an extension of this study.

Summary

The studies in this section show that the predominant R.F.C. is lymphocytic. The evidence favours its non-immune nature. Such an identification does not however classify the lymphocyte as T or B. 44% of the total lymphocytic R.F.C. have features consistent with activated T lymphocytes. The remainder cannot be definitively identified as T or B.

The form of contact between lymphocytic R.F.C. and erythrocyte was predominantly of a pointlike nature, little deformation of attached red cells occurred.

Monocytes were the only other type of cell encountered with any frequency. Their prime distinguishing feature was the deformation of attached red cells which they caused.

CHAPTER V

DISCUSSION OF THE ROLE OF
THE HOMOLOGOUS AND
AUTOLOGOUS ROSETTING CELL
IN THE PATHOGENESIS OF
AUTO-IMMUNE HAEMOLYTIC
ANAEMIA

Contents

1. Prelude
2. T-lymphocyte subpopulations.
 - a) Helper T-lymphocytes.
 - b) Suppressor T-lymphocytes
 - i) Suppressor T-lymphocytes in animals
 - ii) Suppressor T-lymphocytes in man.
3. Auto-Immunity.

1. Prelude

The evidence presented in this thesis, in Chapters III and IV, indicates that the rosette forming lymphocyte studied in auto-immune haemolytic anaemia is a T cell. This conclusion is supported by clinical, experimental and ultrastructural studies.

The ultrastructural features of the rosetting lymphocytes revealed that 62, from a total of 144, had either microfilaments or an area of clearing under the plasma membrane, features which in mouse lymphocytes are found in activated T cells (Matter et al., 1972) and it has been suggested by other investigators, that the ability to form homologous rosettes is a characteristic of activated T lymphocytes (Sheldon and Holborow, 1975a).

The clinical studies of patients with auto-immune haemolytic anaemia in this thesis, have shown, that the number of rosetting cells identified in the peripheral blood correlates, in a positive fashion, with the activity of the disease.

The possible functions of these cells, in A.I.H.A., identified by their ability to form homologous and autologous rosettes, are now discussed in terms of recent work concerning T lymphocyte subpopulations. Such investigations have to date been performed predominantly/

predominantly in animals, and a role for one of these subpopulations, the suppressor T cell, has been postulated in auto-immune disease (Allison et al., 1971).

2. T Lymphocyte subpopulations

There is evidence to support the general concept of subclasses of T lymphocytes in mice. Much of the work has been concerned with differentiation antigens, called Ly antigens, which are found on thymocytes and a proportion of peripheral lymphocytes. There are three such antigens (Ly 1,2,3), all three determinants are found on 85 to 90 percent of thymocytes. Lymphocytes with a differing pattern of Ly determinants are found peripherally (Cantor and Boyse, 1975; Kisielow et al., 1975). These authors have shown that depletion of cell populations, carrying differing Ly antigens, abolishes certain T lymphocyte functions from the remaining subpopulation. Thus helper or cytotoxic activity can be selectively removed, with the use of specific antisera.

Three functional subpopulations have been identified, T helper cells (Ly 1), T cytotoxic cells and T suppressor cells (Ly 2, 3). Immature T cell precursors, like thymus cells, carry all three/

three Ly antigens.

Anti-Ly antisera are not generally available, but T lymphocyte subpopulations can be identified in other ways. T lymphocytes, in the mouse, carry differing quantities of Θ -antigen, and varying effects of adult thymectomy and anti-thymocyte serum on functional aspects of T lymphocytes can be demonstrated (Janeway et al., 1975).

In auto-immune haemolytic anaemia it is the manufacture and secretion of antibody, by B-lymphocytes and their progeny, which leads to sensitisation of the red cell and its final destruction, predominantly by tissue macrophages. Although it is not possible to exclude a role for the cytotoxic T lymphocyte in A.I.H.A., it seems more likely that disturbances in the other subclasses of T cell would be of more importance in the pathogenesis of this disorder. Thus interactions of a co-operative, or suppressive nature between lymphocyte subpopulations might be abnormal in auto-immune disease, and be a crucial factor in the underlying cellular immune disturbance leading to disease. A brief review of helper and suppressive T lymphocytes is now presented. Although in this thesis the rosetting cells have not been identified on a functional basis, such a review indicates a suitable direction for future research.

a)/

a) Helper T lymphocytes

There is a large literature on this aspect of T - B cell interaction and it has recently been reviewed (Greaves et al., 1974a; Playfair, 1974). Co-operation was first suggested by the experiments of Claman et al. (1966), which showed that irradiated mice, given both thymus and bone marrow cells, made a far greater primary response to sheep erythrocytes, than recipients of either thymus or marrow cells alone. It is now accepted that T cell help is needed for most IgG, and some IgM antibody responses (Gershon and Paul, 1971).

The actual mechanism by which T cells exert this effect is not known, but experiments carried out in vitro have detected the presence of several 'soluble factors' which can mediate a co-operative response. The most outstanding studies have been those of Feldmann (1972), who showed that when T and B lymphocytes are cultured, in separate compartments together with antigen, a co-operative response is mediated between them by a soluble product from the T cell compartment. This product acts not directly but via the surface of macrophages.

Such co-operation both in vitro and in vivo has been demonstrated for a variety of antigens. In all cases it is the B lymphocyte which secretes/

secretes the antibody.

Recently it has been shown that helper T-cells express specific antigen binding receptors (Konttinen and Andersson, 1975). This is of some importance, as similar specificity has not been demonstrated satisfactorily for suppressor cells, the next class of cell to be described. Lack of demonstrable specificity has been used by Playfair (1974) as an argument against the importance of such a subpopulation.

There is now evidence that T-B cell co-operation is not necessary for all humoral immune responses. There are several well delineated thymus-independent antigens which do not appear to require T-cell help (Greaves et al., 1974a).

b) Suppressor T lymphocytes

The role of the suppressor T cell in the aetiology of auto-immune disease has recently been emphasised (Allison et al., 1971), and two reports have recently described suppressor T cells in human lymphocyte populations (Waldmann et al., 1974; Sampson et al., 1975). In view of this, some of the evidence for the existence of this cell and its characteristics are now presented.

i)/

i) Suppressor T cells in animals

Although Gershon and Kondo (1970) were the first to suggest that T lymphocytes were involved in the suppression of an immune response, Gershon himself (1975), maintains that in mice, the suppressor T cell has not passed from a concept to an entity. Despite that latter statement, suppression as a mechanism of control has been demonstrated in various systems, e.g., immunoglobulin allotype synthesis suppression (Jacobson et al., 1972), and as a mechanism of suppression in tetraparental mice (Phillips and Wegmann, 1973).

Many other authors have presented evidence to support the existence of the suppressor T lymphocyte. Baker (1975) showed that the response to type III pneumococcal polysaccharide (a thymus independent antigen), was significantly increased by prior treatment with anti-lymphocytic serum. It has also been shown that thymocytes or spleen cells from rats, hyperimmunised with an antigen, could rapidly suppress an ongoing antibody response in another rat against the same antigen (Tada et al., 1975). The same authors showed that the suppressor activity was abrogated by prior treatment with anti- θ serum. In addition, T lymphocytes can be non-specifically activated to actively suppress antibody/

antibody production by B lymphocytes (Rich and Pierce, 1973), and responses to both thymus dependent, and thymus independent antigens can be affected.

Such suppressive activity in mice can be reduced by treatment of the cell donors with hydrocortisone (Nachtigal et al., 1975). As mature suppressor T cells were shown to be hydrocortisone resistant, the authors postulated that this drug acted on T suppressor cell precursors and gave evidence to support this view.

In addition to suppression of B lymphocyte activity by T cells, Gershon et al. (1974) have recently demonstrated suppression of other T-lymphocytes and of macrophages.

Further clarification is required concerning the target of suppressor activity. In delayed hypersensitivity, T-cells themselves, can suppress other T cells (Gershon et al., 1974). In the formation of antibodies it is not known whether suppression is exerted directly on B-cells, via macrophages, or on other T-cells that would have facilitated the response of B cells.

ii) T-Suppressor Cells in humans

There are two reports which claim to have demonstrated human suppressor cells. The first was by Waldmann et al. (1974) who observed that/

that co-culturing peripheral blood lymphocytes or isolated T cells from hypo-gammaglobulinaemic patients, with normal lymphocytes, led to suppression of immunoglobulin synthesis by these normal lymphocytes. They suggested, that the human disease might be caused or perpetuated by an abnormality of regulatory T cells, which act to suppress B cell maturation and antibody production.

The only other report in humans is that of Sampson et al. (1975), who claimed to have demonstrated suppressor cells in human splenic lymphocyte suspensions. The model they used, was prior incubation of the lymphocytes under test with phytohaemagglutinin, and assayed the subsequent ability of these cells to inhibit a mixed lymphocyte reaction.

They found non-specificity of the suppressor effect and that suppression was dependent on cell dose. Only at high cell dosage was there inhibition of the mixed lymphocyte reaction.

These two reports are the only ones I have encountered, which directly demonstrate suppressive effects in human lymphocyte systems. Despite the paucity of strong evidence in man, it seems likely to me, that over the next few years there will be more definitive and convincing demonstrations of suppressor/

suppressor T cells in humans. What part could such cells with suppressive activity play in the pathogenesis of auto immune haemolytic anaemia?

3. Auto-Immunity

Despite many recent theoretical models (Bretscher, 1973; Coutinho and Möller, 1975; Weigle, 1971; Grabar, 1974; Stiller et al., 1975; Allison et al., 1971; Allison, 1971; Micklem, 1971), there is little definitive work on the cellular changes responsible for the initiation of auto-immune reactions, especially in man.

Natural self-tolerance is the hallmark of the immune system, and the mechanism by which tolerance is maintained may shed light on the pathogenesis of auto-immune disease.

Tolerance in this context means the lack of immune response to an antigen, following exposure to that antigen, by an animal able to respond normally to other antigens. An individual is therefore operationally tolerant to his own antigens, and tolerance can in fact be experimentally induced.

Paul Ehrlich (1906) was the first to draw attention to the fact that vertebrates do not commonly make antibodies against their own body constituents/

constituents, with his term 'horror autotoxus'. Theoretically, non-reactivity to self-antigens can be attributed to either of two basic mechanisms. Firstly, potentially self-reactive lymphocytes might be eliminated during embryonic development as suggested by Burnet (1959). This means that lymphocytes reactive with accessible auto-antigens should not be demonstrable in normal animals.

A second explanation, for which there has been increasing evidence recently, is that auto-reactive lymphocytes exist, but that control mechanisms actively inhibit the expression of these self-reactive cells. Self-reactive B lymphocytes do normally exist (Coutinho and Möller, 1975) but auto-antibodies are not normally produced to any considerable extent. These auto-reactive lymphocytes have been demonstrated to thyroglobulin, in patients with human immune thyroiditis (Perrudet-Badoux and Frei, 1969), and to thyroglobulin in normal people (Bankhurst et al., 1973).

Similar thyroglobulin binding cells have been demonstrated in the thymus, with highest levels in the foetus which decrease progressively with age (Roberts et al., 1973). Comparable findings have been reported for human encephalitogenic protein in peripheral blood and lymph node cell suspensions (Yung et al., 1973).

In/

In animals too, the evidence indicates that self-reactive cytotoxic lymphocytes exist, which bear receptors specifically recognising self antigens (Cohen and Wekerle, 1973; Wekerle et al., 1973). In particular in animal work, auto-reactive cells which produce antibody against red cell constituents, have been demonstrated in normal mice (Cunningham, 1974), and in mice under strong antigenic stimulation, such as the injection of bacterial vaccines (Klemparskaya, 1973). Strong antigenic stimulation of this nature, has been shown to terminate experimentally induced tolerance (Chiller and Weigle, 1973).

An earlier report indicated, that a proportion of apparently healthy old mice (47%) possessed significantly greater numbers of spleen cells capable of auto-antibody production, than middle aged or young mice (Hildemann and Walford, 1966). This observation accords with more recent work in the New Zealand Black (NZB) strain of mouse which develops auto-immune disease, including auto-immune haemolytic anaemia. At corresponding ages, NZB mice have a hundredfold more auto-antibody producing cells than other strains of mice. These mice therefore achieve humoral and cellular immunological maturity when still very young/

young (Talal and Steinberg, 1974).

Thus it seems clear that auto-reactive cells exist, two methods of control to prevent the expression of such auto-reactivity have been postulated. Hellström and Hellström (1970) suggested that some forms of tolerance were effected by antibodies (blocking antibodies) which suppressed antigen recognition by immunologically competent cells. Other authors (Hellström et al., 1971; Bansal et al., 1973; Wekerle et al., 1973) have suggested that tolerance in some systems is due to serum factors, which are either tolerogenic antigen, or enhancing antibodies, or antigen-antibody complexes.

The second mode of control postulated is a specific feedback by T lymphocytes (suppressor cells) on the production of auto-antibody by immunocompetent B lymphocytes (Allison et al., 1971; Allison, 1971; Jacobson et al., 1972). The evidence for the existence of, and the characteristics of this cell have been previously delineated.

Much of the experimental support for the importance of the suppressor T-lymphocyte in auto-immune disease is dependent on work in NZB mice which has recently been reviewed (Talal and Steinberg, 1974).

In/

In this review, the authors state that the immune status of NZB mice is characterised by an imbalance, in which B-cell activity and antibody responses are excessive, and T-cell activity is depressed. Such cellular abnormalities in NZB mice are explainable by a progressive loss of thymic function with age. Certainly in NZB mice, auto-immune disease is accelerated by neonatal thymectomy, as it is, but less dramatically, in normal mice (Yunis et al., 1967). This phenomenon can be prevented in NZB mice by implanting thymic grafts from two week old NZB mice but not from ten week old mice.

The authors suggest that the loss of suppressor T-cells may help to explain the humoral hyper-responsiveness of NZB mice.

Charrière and Bach (1974), recently reported a dramatic effect of adult thymectomy on the numbers of autologous rosetting cells in mice, the latter increasing twenty-fold, three months following this operation. In man, these authors found that aged subjects had more autologous rosetting cells than younger ones, and suggested, that a study of autologous rosetting cells, would represent an interesting model for the study of human auto-immune disease.

I contend that further study of the lymphocytic homologous rosetting cell found in auto-immune haemolytic anaemia, especially with regard to its function, would be an extremely fruitful area of research. This cell could of course be a helper or suppressor T cell, and assay systems similar to those described by Waldmann et al. (1974) or Sampson et al. (1975) would be capable of detecting both effects. The use of such an assay system would not, however, be limited to the study of A.I.H.A. but could be extended to other areas, leukaemias, lymphomas and other malignant disorders in which excessive suppressive effects or their loss may play an important role.

Miller (1975) states that the suppressive effect of T cells on B cell responsiveness, and on other T-cell activities must be considered in the pathogenesis of auto-immune diseases. Further understanding of the mechanism of action of suppressor T cells, might conceivably lead to the control of some of these disorders.

If the concept of immunoregulation of auto-immunity by T-cells is realistic, then the treatment of auto-immune disease with immunosuppressive drugs may indeed be a two-edged sword.

CHAPTER VI

THE EFFECT OF CORTISONE
ACETATE ON MURINE
LYMPHOCYTE SUBPOPULATIONS,
MONOCYTES, AND THE BLOOD
CLEARANCE OF ANTIBODY
SENSITISED ERYTHROCYTES.

1. Background
2. Corticosteroids and human lymphocytes
3. Corticosteroids and human monocytes
4. Organ sequestration of sensitised erythrocytes
in humans
5. Conclusions
6. Plan of the work in this chapter.

1. Background

Since they were introduced in 1951 (Damashek et al., 1951), corticosteroids have been the major therapeutic tool in the treatment of auto-immune haemolytic anaemia. In one early series, about 90% of patients responded partially or totally to the institution of such therapy (Damashek and Komninos, 1956), but some 65% relapsed following discontinuation of treatment. An improvement in the patient with such therapy usually occurs within 2 - 21 days, with a median time of 9 days (Pirofsky and Bardana, 1974).

The mechanism underlying this high response rate is quite unknown. There have been many proposals, including the reduction of anti-erythrocytic antibody (Allgood and Chaplin, 1967), decrease of antigen-antibody interactions (Rosse, 1971) and inhibition of clearance of antibody coated erythrocytes by fixed macrophages of the mononuclear phagocyte system (Rosse, 1971).

With the development of techniques enabling identification of subpopulations of lymphocytes (Greaves, et al., 1974a), increased interest has been directed to the effect of corticosteroids on these cells in animals and man.

One/

One purpose, of the investigations in Chapter III, was to find whether corticosteroid therapy had an effect on the blood levels of homologous and autologous rosetting cells. Because of the small number of patients involved and the variability of dosage employed, this could not be determined.

In this chapter, a study of the changes in lymphocyte subpopulations and monocyte function in mice following corticosteroid therapy is presented.

What follows, in this introductory section, is a review of the known effects of corticosteroids on lymphocytes and monocytes, and on blood clearance of IgG sensitised erythrocytes in man. I feel this is necessary, as much of the work in this thesis is concerned with human leucocytes. Such a review, though aiding the interpretation of animal results and their application to man, emphasises the difficulties of such interspecies comparison. For example, a major difference exists with regard to human lymphocytes and similar cells in the mouse, lymphocytes in humans being much more resistant to the effect of steroids (Claman, 1975).

2. Corticosteroids and human lymphocytes

Conflicting evidence exists with regard to the effect of corticosteroids on human lymphocyte subpopulations/

subpopulations but there are two areas in which most authors are agreed. Firstly, circulating T lymphocytes in vivo, are more adversely affected than are B lymphocytes (Yu et al., 1974; Bach et al., 1975). Secondly, the differentiated descendants of B lymphocytes, such as plasma cells and their neoplastic counterparts, are resistant to the action of glucocorticosteroids (Baxter and Harris, 1975).

For example, T lymphocytes have been shown to fall after a single injection of steroid in man (Bach et al., 1975). This fall has been shown to be due to a shift from the circulation into an extravascular pool (Fauci and Dale, 1975). A similar fall has recently been demonstrated in patients with renal transplants receiving high dose steroid (Tursz et al., 1976). There is some evidence that the extravascular pool may consist, at least in part, of the bone marrow (Borella and Green, 1972).

In the human, the majority of thymus cells form sheep erythrocyte rosettes which are stable at 37°C and resist vigorous shaking, these properties contrast with peripheral T lymphocytes. Galili and Schlesinger (1975) have presented evidence in man, showing that stable rosetting cells decrease with advancing age and that steroid treatment results in/

in a reduction of these cells in the thymus. The lymphocytes remaining in the thymus following this therapy, resemble peripheral T lymphocytes. These authors suggested that the human thymus contains a minor population of immunocompetent cells, resistant to corticosteroids, and differing from the majority of thymus cells by their failure to form stable sheep cell rosettes. Confirmation of these results would mean that human thymic cells resemble those of the mouse thymus in their response to corticosteroid therapy (Blomgren and Andersson, 1970), which will be detailed in the discussion.

The absolute numbers of circulating B lymphocytes are reduced by steroid therapy (Yu et al., 1974). As such cells are not easily lysed in vitro by steroid (Schreck, 1961), it is likely that the fall demonstrated, is due to a shift of lymphocytes from the intravascular compartment, though there is no published evidence on this point.

There is conflicting evidence with regard to the effect of corticosteroid on the production of immunoglobulins by B lymphocytes and their progeny. Griggs et al. (1972) were unable to demonstrate a change in serum IgG levels in patients on prednisone therapy, though they did show a shortened survival of IgG. In contrast, Tuchinda et al. (1972) demonstrated reduced serum immunoglobulin levels in normal volunteers given methyl-prednisolone.

3. Corticosteroids and human monocytes

There is very little published work on this topic. The effect of corticosteroids on the mononuclear phagocyte system have usually been assessed indirectly, by the changes induced by such drugs, on the clearance from the blood of antibody sensitised erythrocytes.

Mollison (1962) demonstrated a reduced rate of clearance of antibody sensitised erythrocytes, 5 to 6 days after cortisone therapy. He concluded that the efficacy of corticosteroids, in preventing clearance of IgG sensitised red cells, was inversely related to the number of IgG antibody molecules coating the red cell. It has been shown more recently, that the administration of corticosteroids results in a rapid decrease in erythrocyte bound antibody, with an increase in free circulating antibody (Rosse, 1971), resulting in a prolongation of erythrocyte survival.

4. Organ sequestration of sensitised erythrocytes in humans

Over the last two decades, reports have related the patterns of organ sequestration to the type of sensitisation of the red cell. Lewis et al. (1960) showed that red cells coated with complement were/

were mainly, but temporarily sequestered in the liver, and this finding was confirmed in cold agglutinin disease by Evans et al. (1968).

Borne et al. (1973) have shown that red cells, from patients with A.I.H.A. associated with incomplete IgG or IgA warm auto-antibodies, are mainly destroyed in the spleen. Certainly patients who have undergone splenectomy have considerably less haemolysis for a given concentration of cell bound antibody, than patients not splenectomised (Rosse, 1971).

Though specific interaction with Fc receptors on splenic macrophages may partially account for this, other factors play a part. The slow circulation in the spleen pulp probably permits red cell auto-agglutination to occur. This results in mechanical trapping of these cells by the slit like stomata which control the exit of cells from the spleen pulp (Dacie, 1970). A further factor, is that red cells coated with incomplete antibodies may be less deformable than normal (Teitel, 1967). The liver acts only as an important haemolytic organ, in relation to IgG antibodies, when the red cells are heavily coated (Dacie, 1970).

5. Conclusions

Human T-lymphocytes are more sensitive to corticosteroid therapy than B-lymphocytes. The effect exerted by a single large dose of corticosteroid on peripheral blood levels, would appear to be due to redistribution rather than lysis. There is little published work on the effect of similar drugs on human monocytes.

6. Plan of the work in this chapter

The Θ -antigen, which is now accepted as a marker of thymus derived lymphocytes in mice (Raff, 1969), has been used to record the effect of corticosteroid on lymphocyte subpopulations in the mouse. The organs studied were the spleen, thymus and lymph nodes.

At the time of maximal cellular depression, as revealed by the first study, blood clearance of IgG sensitised mouse erythrocytes was measured, together with an assessment of splenic and hepatic sequestration.

Finally in vivo and in vitro studies of the effects of corticosteroids on murine monocytes are presented. It was hoped that the results of these investigations in the mouse, taking into account the reservations mentioned, might indicate a suitable/

suitable mode of investigation in human auto-immune haemolytic anaemia.

Although it was hoped to study homologous and autologous rosetting cells in the mouse peripheral blood and tissues, the levels found, were too low for any effect to be meaningful. This aspect of the investigation therefore had to be abandoned.

RESULTS

1. The effect of cortisone acetate on murine lymphocyte numbers and subpopulations.
 - a. Spleen
 - i) Total cell number
 - ii) Absolute B lymphocyte and T lymphocyte numbers.
 - iii) T/B lymphocyte ratio
 - iv) T/B lymphocyte ratio following continuous steroid therapy.
 - b. Thymus
 - i) Total cell number
 - ii) Percentage of cells carrying Θ -antigen
 - c. Lymph nodes
 - i) Total cell number
 - ii) T/B lymphocyte ratio.

2. The effect of cortisone acetate on murine monocytes.
 - a. In vitro addition to macrophage monolayers
 - b. In vivo effect
 - c. Combined in vivo and in vitro effects

3. The effect of cortisone acetate on the blood clearance of normal and IgG sensitised mouse erythrocytes.
 - a. Blood clearance in normal mice.
 - b. Blood clearance in cortisone treated mice.

4. The effects of cortisone acetate on splenic and hepatic sequestration (total organ).

5. The effects of cortisone acetate on splenic and hepatic sequestration (per unit weight).

Results

1. The effect of cortisone acetate on murine lymphocyte numbers and subpopulations.

a. Spleen

i) Total cell number

The total number of cells in the spleen was reduced for a period of 10 days following the cortisone treatment (Fig. 1). There was fluctuation in the degree of reduction, with a rapid return to normal cell numbers 10 days after injection.

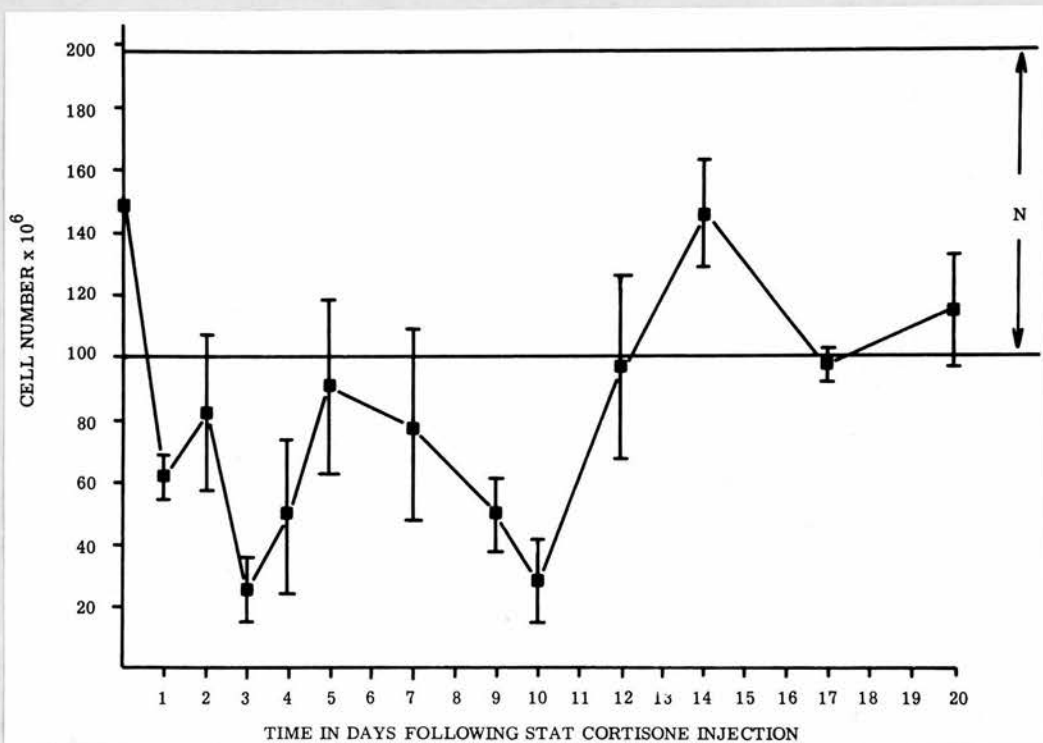


FIGURE 1 Nucleated cell numbers in mouse spleen following cortisone acetate injection. The area between the horizontal lines (N) represents the mean \pm two standard deviations, measured in 43 control mice. Each symbol represents the mean \pm two standard deviations in at least four test mice. These two stipulations apply for the following eight figures, apart from the differing numbers of controls, which will appear in brackets in the legend.

ii) Absolute B-lymphocyte and T-lymphocyte numbers

Cortisone acetate caused a reduction in the absolute numbers of B-lymphocytes for 12 days (Fig. II). The effect on T-lymphocytes was not as clearcut (Fig. III).

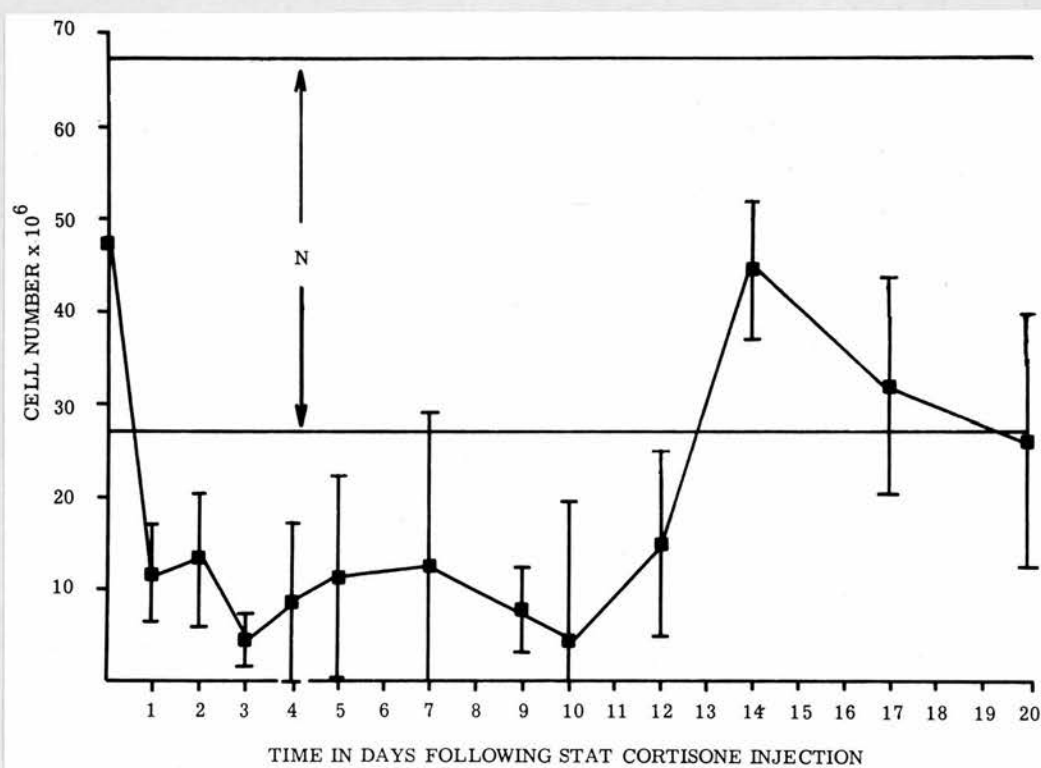


FIGURE II B-lymphocyte numbers in mouse spleen following cortisone acetate (33 control mice).

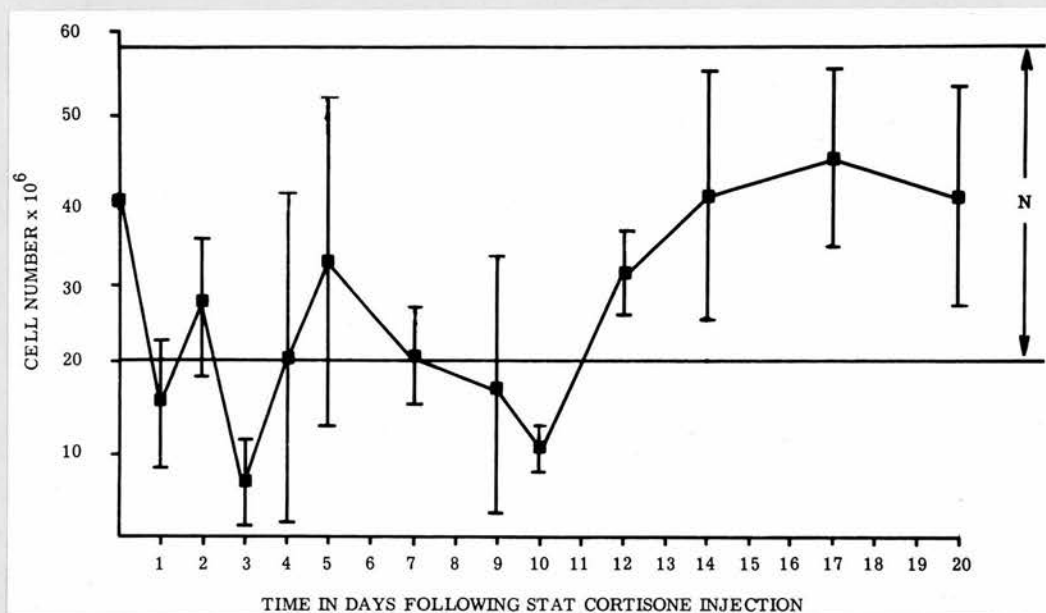


FIGURE III T-lymphocyte numbers in mouse spleen following cortisone acetate (33 control mice).

iii) T/B lymphocyte ratio

A differential effect was seen (Fig. IV). For 10 days, there was an elevation in the ratio, indicating a greater sensitivity of splenic B-lymphocytes than T-lymphocytes, to cortisone acetate.

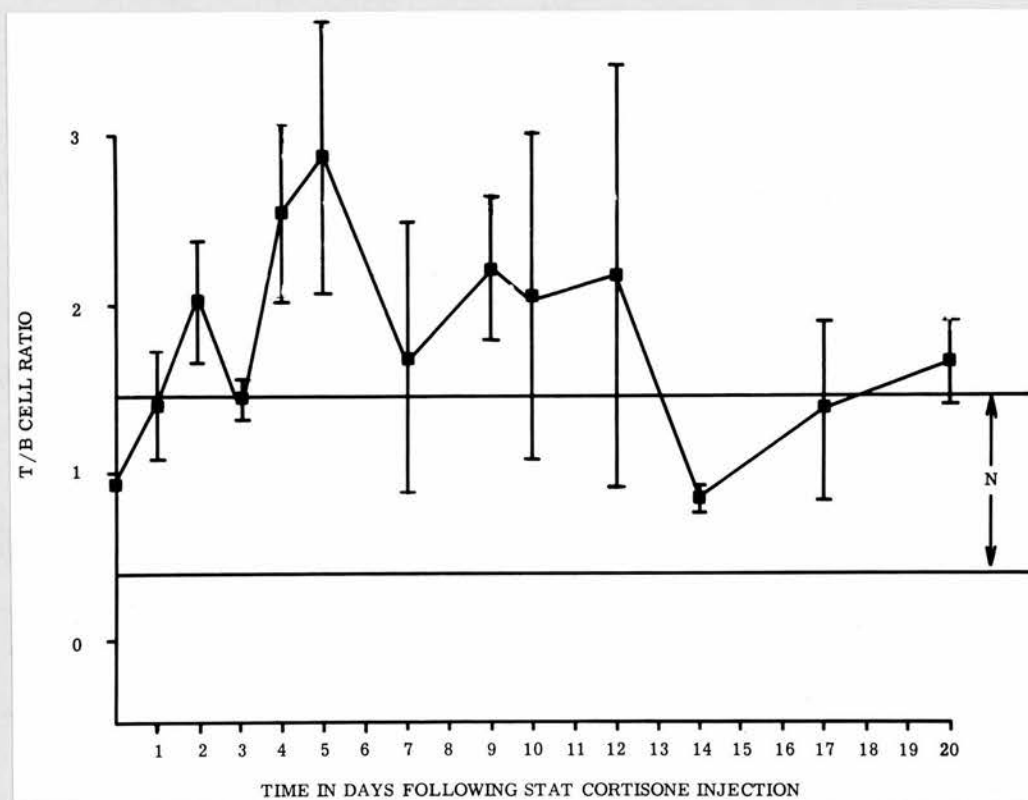


FIGURE IV T/B lymphocyte ratio in mouse spleen following cortisone acetate (33 control mice).

iv) T/B lymphocyte ratio following continuous steroid therapy

This experiment magnified the differential effect (Fig. V). It was not possible to carry the experiment beyond 10 days, as many mice died after this period.

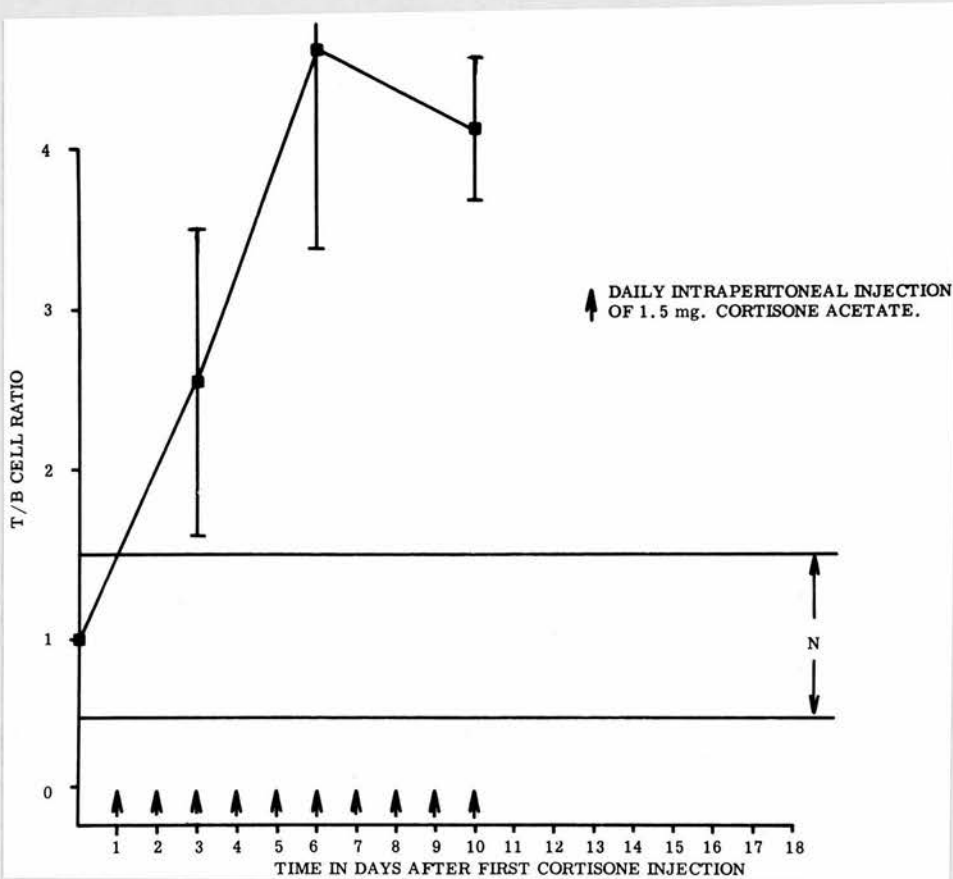


FIGURE V T/B lymphocyte ratio in mouse spleen following continuous cortisone acetate (33 control mice).

b. Thymus

i) Total cell number

The total number of cells was reduced to 2% of its original value, three days following treatment, and remained at this low level for 10 days. There was a rapid increase in cell number thereafter, though at the end of the observation period, the cell number had not returned completely to normal (Fig. VI).

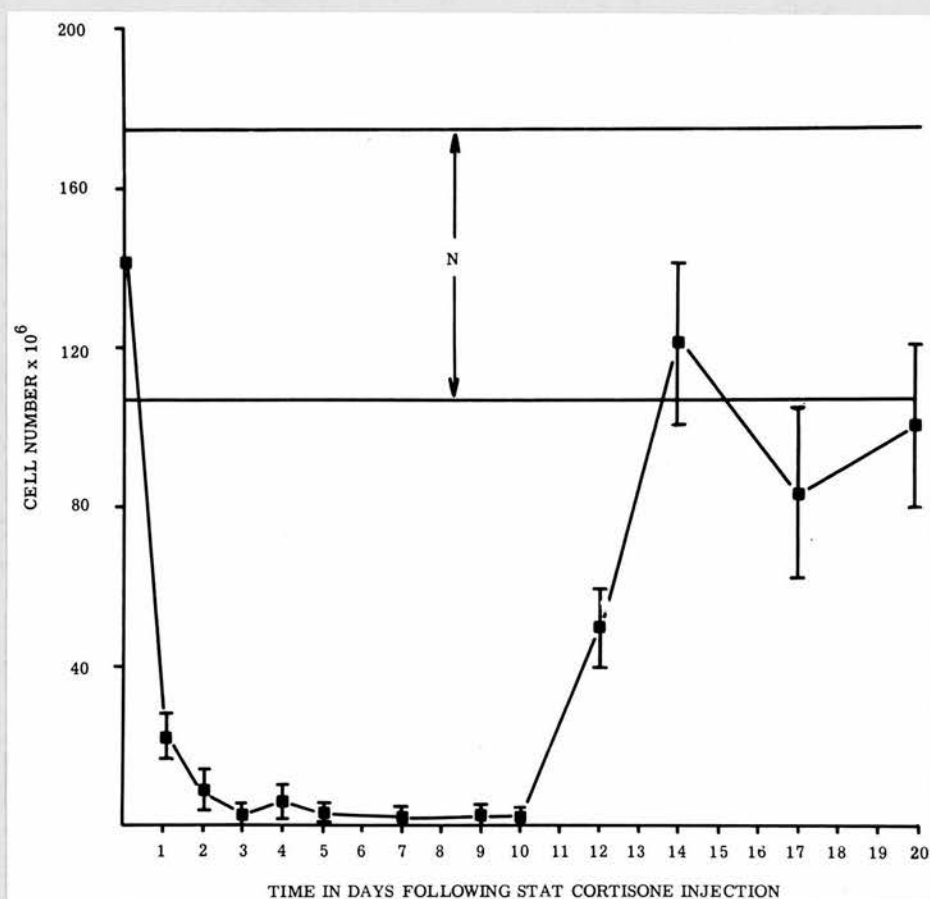


FIGURE VI Thymic cell number following cortisone acetate injection (43 control mice).

ii) Percentage of cells carrying the
Θ-antigen

The percentage of lymphocytes carrying the Θ-antigen decreased following cortisone acetate, being maximally depressed at day 10. There was a rapid return to normal thereafter (Fig. VII).

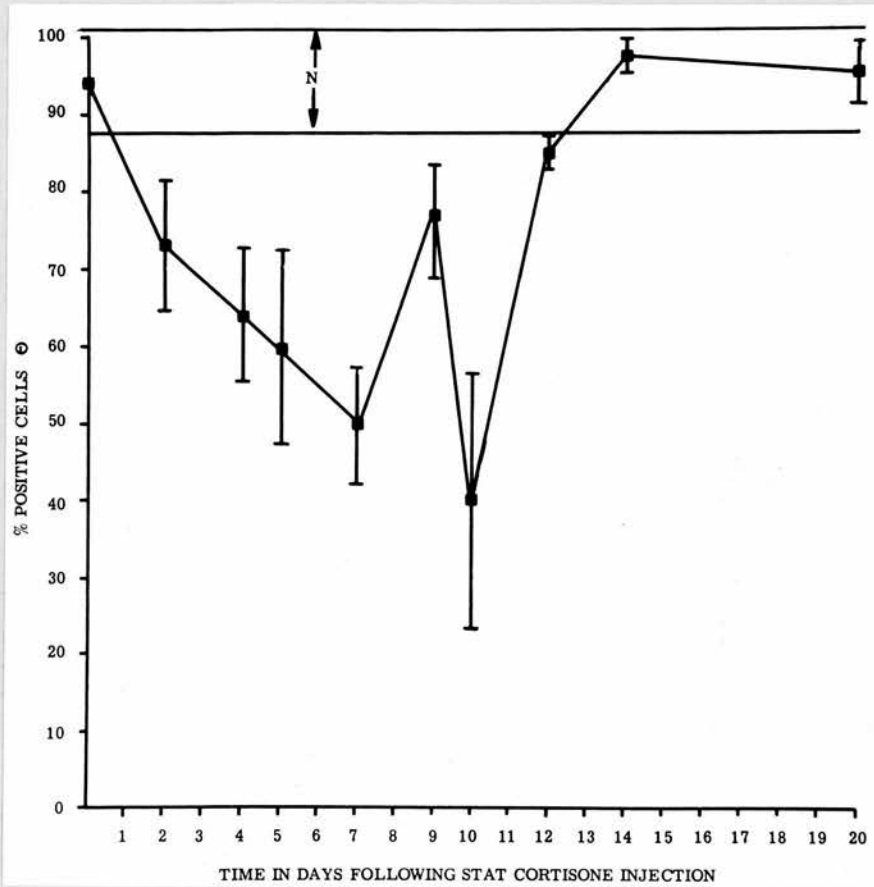


FIGURE VII Percentage of lymphocytes carrying the Θ -antigen in the thymus following cortisone acetate (33 control mice).

c. Lymph Nodes

i) Total cell number

The total cell numbers were reduced to 17% of the initial population, two days following treatment, and remained depressed for the period of observation (Fig. VIII).

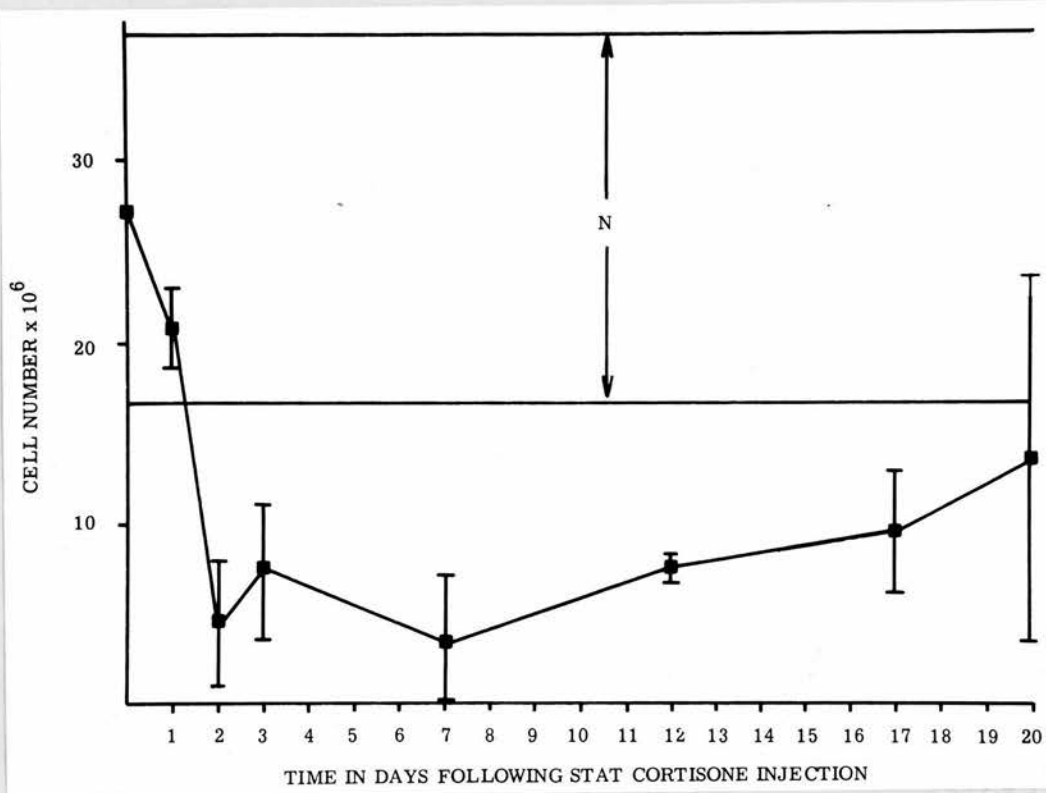


FIGURE VIII Lymph node cell number following cortisone acetate injection (18 control mice).

ii) T/B lymphocyte ratio

There was a rise in this ratio, which remained elevated for the period of observation (Fig. IX).

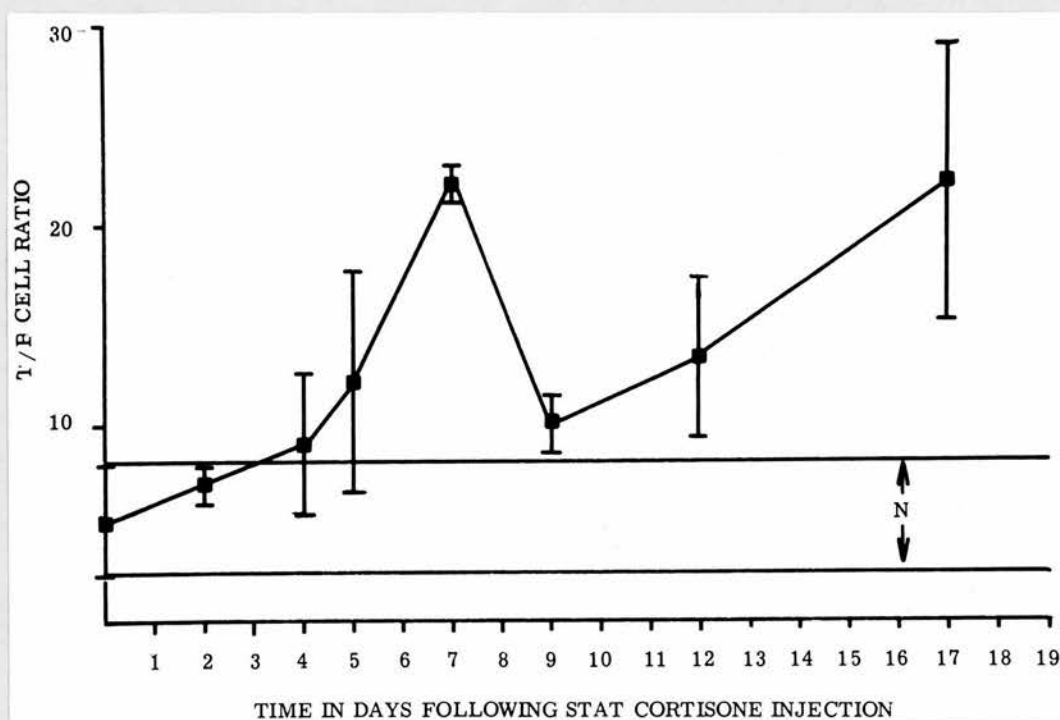


FIGURE IX T/B lymphocyte ratio in mouse lymph nodes following cortisone acetate (18 control mice).

2. The effect of cortisone acetate on murine monocytes

a. In vitro addition to macrophage monolayers

A representative experiment (four were carried out) is shown in Fig. X. Unsensitised sheep erythrocytes were not ingested. The increased phagocytosis, induced by IgG sensitisation, was inhibited by the two higher doses of cortisone added to the monolayers.

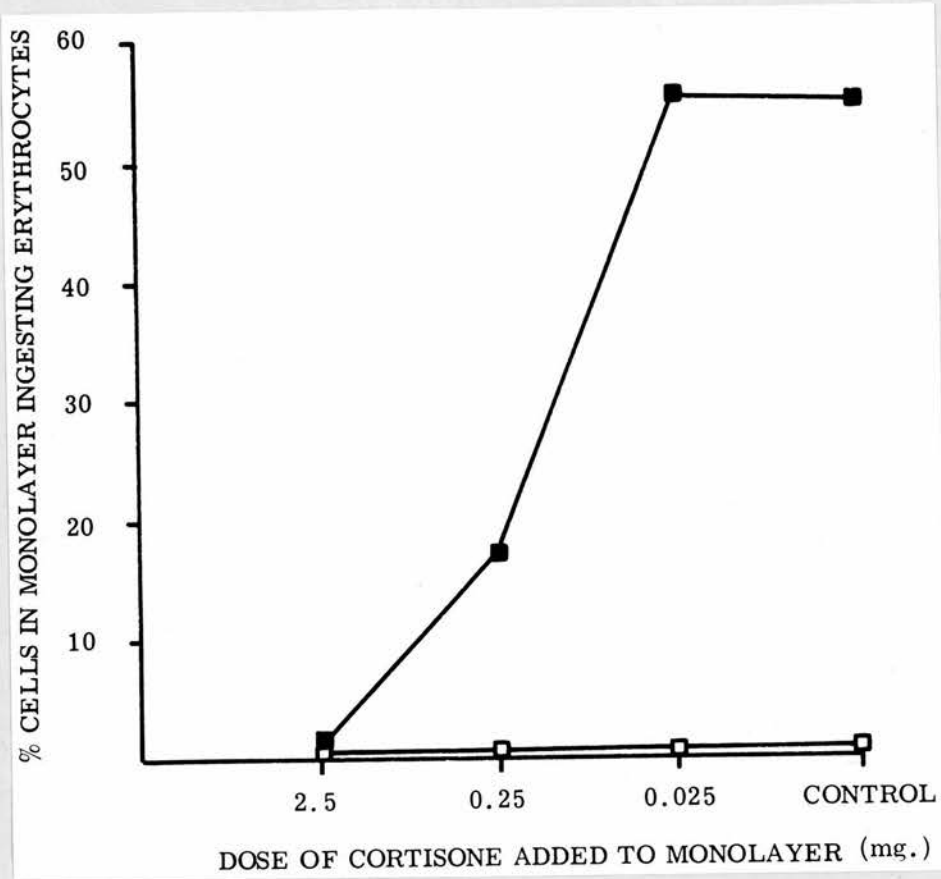


FIGURE X The effect of addition of cortisone acetate, in varying dosage, on the ability of murine peritoneal macrophage monolayers to ingest sheep erythrocytes (■ = IgG sensitised erythrocytes □ = unsensitised erythrocytes).

b. In vivo effect

In vivo administration of cortisone acetate, had no effect on the subsequent phagocytic potential of peritoneal macrophages derived from such treated mice (Fig. XI). Two experiments were performed.

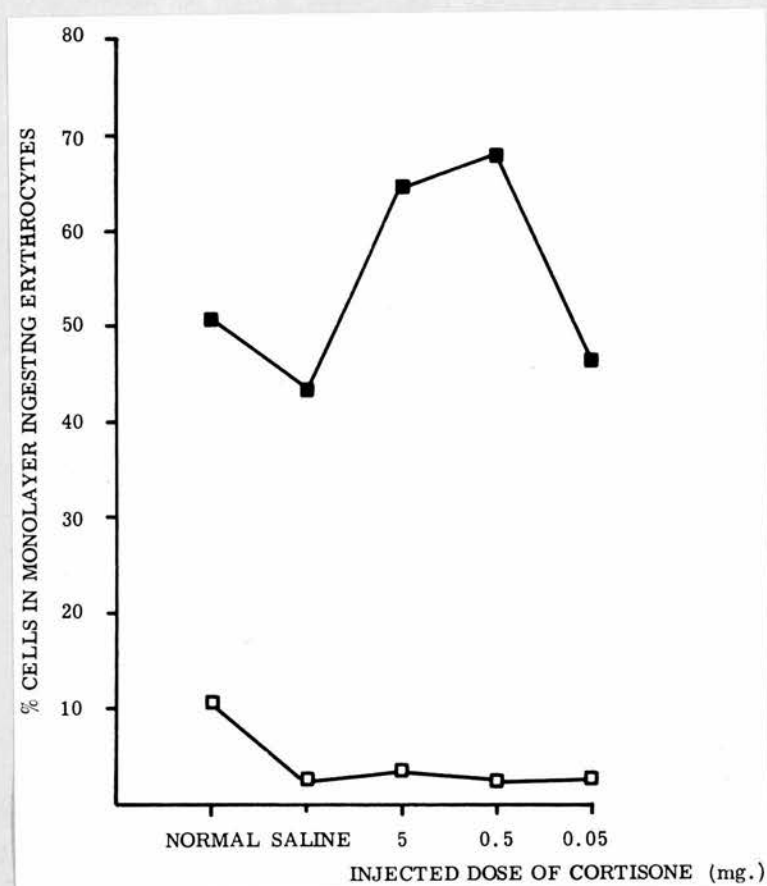


FIGURE XI The effect of in vivo administration of cortisone acetate for three days, on the phagocytic potential of peritoneal macrophage monolayers (■ = IgG sensitised sheep erythrocytes. □ = unsensitised erythrocytes).

c. Combined in vivo and in vitro effects

The addition of cortisone acetate in vitro, to macrophage monolayers derived from cortisone treated mice, gave similar results to that of the first experiment in this small series. Thus higher doses of in vitro cortisone resulted in higher inhibition of phagocytosis (Fig. XII).

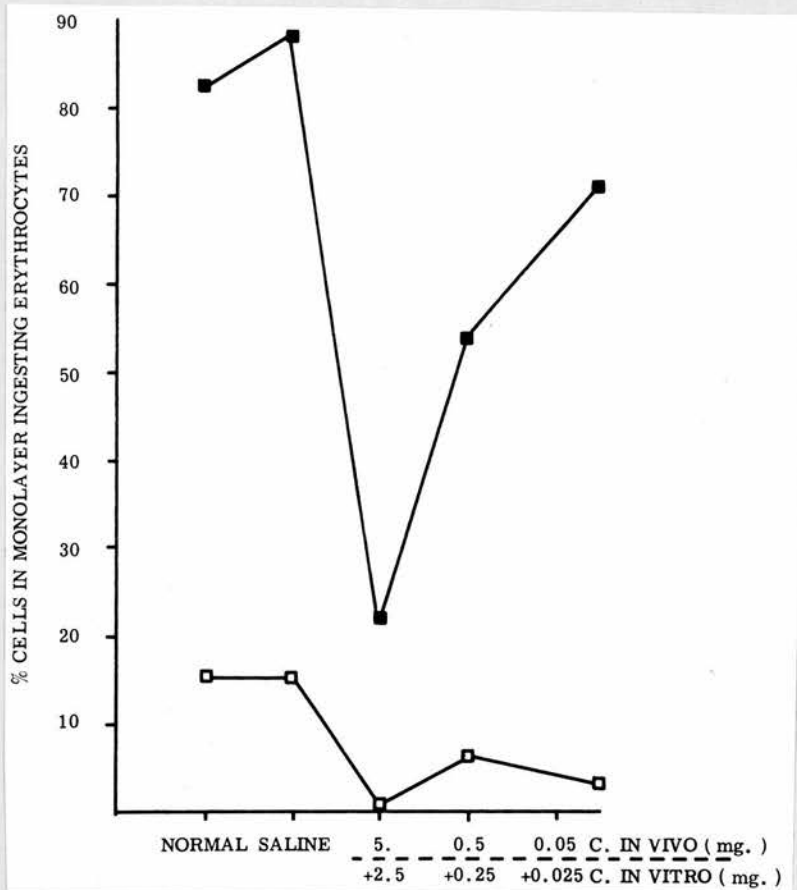


FIGURE XII The effect of combined in vivo (3 days) and in vitro cortisone acetate, on the ability of murine peritoneal macrophage monolayers to ingest sheep erythrocytes (■ = IgG sensitised erythrocytes □ = unsensitised erythrocytes. C = cortisone acetate).

3. The effects of cortisone acetate on the blood clearance of normal and IgG sensitised mouse erythrocytes

a. Blood clearance in normal mice

The K value for normal red cells was 0.000125. There was accelerated clearance of IgG sensitised red cells at all levels tested. There was/

was no difference in the rate of clearance of 1/1500 and 1/1750 sensitised erythrocytes ($k = 0.00054$ and 0.00055 respectively). Clearance of cells sensitised at a level of 1/1250 was more rapid ($k = 0.00078$). The clearance slopes are illustrated in Fig. XIII.

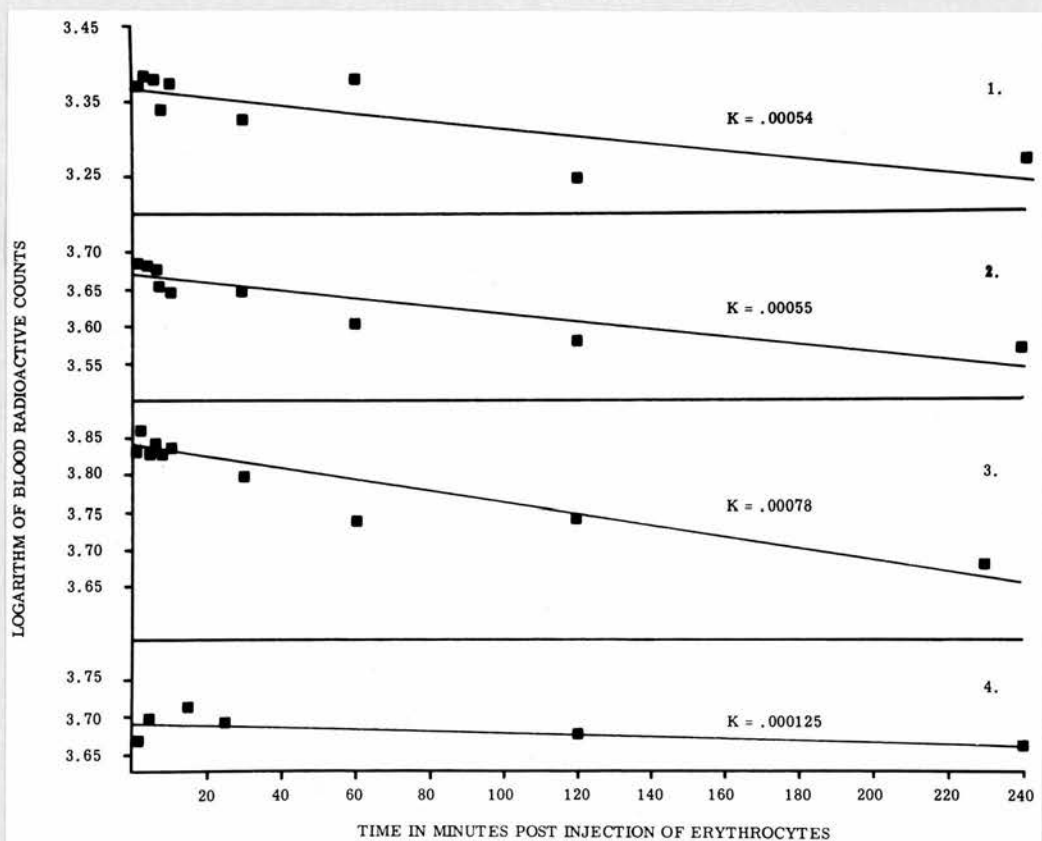


FIGURE XIII Blood clearance slopes of normal and IgG sensitised erythrocytes (at three different levels) in normal mice. K = clearance index. Slope 1 = clearance of 1/1750 sensitised erythrocytes. Slope 2 = clearance of 1/1500 sensitised erythrocytes. Slope 3 = clearance of 1/1250 sensitised erythrocytes. Slope 4 = clearance of normal erythrocytes.

b. Blood clearance in cortisone treated mice

The/

The blood clearance rate of erythrocytes sensitised at 1/1500 was halved, by prior treatment of the mice with cortisone acetate (Fig. XIV).

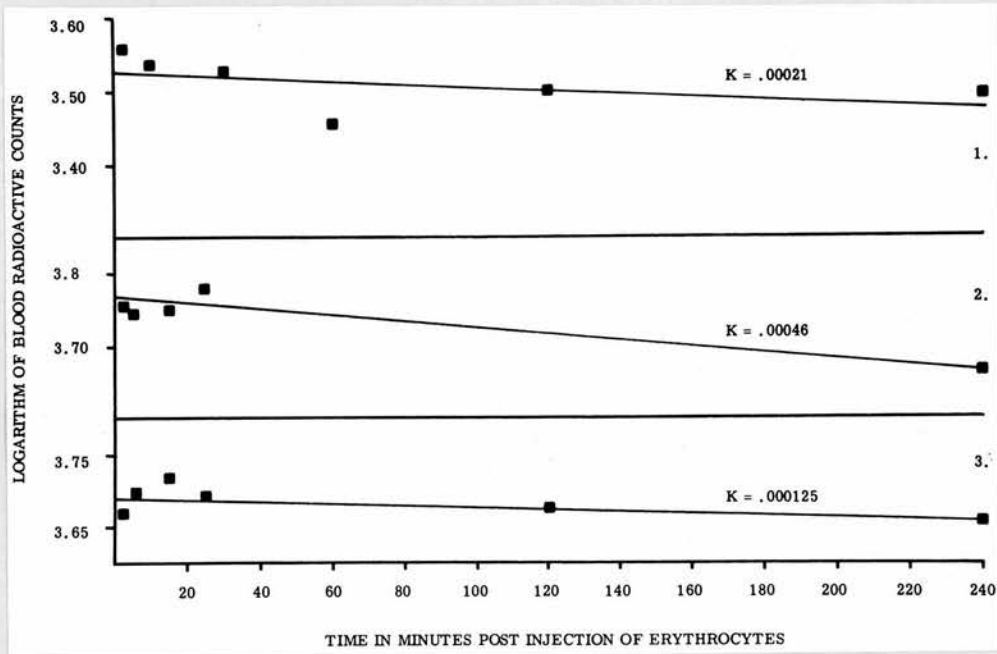


FIGURE XIV Blood clearance slopes of normal and IgG sensitised erythrocytes (1/1500 only) in cortisone acetate and untreated mice. K = clearance index. Slope 1 = clearance of 1/1500 sensitised erythrocytes in cortisone treated mice. Slope 2 = clearance of 1/1500 sensitised erythrocytes in normal mice. Slope 3 = clearance of normal erythrocytes in normal mice.

4. The effects of cortisone acetate on the splenic and hepatic sequestration of normal and IgG sensitised erythrocytes, expressed as the percentage of the injected dose found in the total organ.

a./

a. Increasing red cell sensitisation

Only with a sensitisation carried out at 1/1000 was there a distinct predominance of hepatic sequestration (Fig. XV).

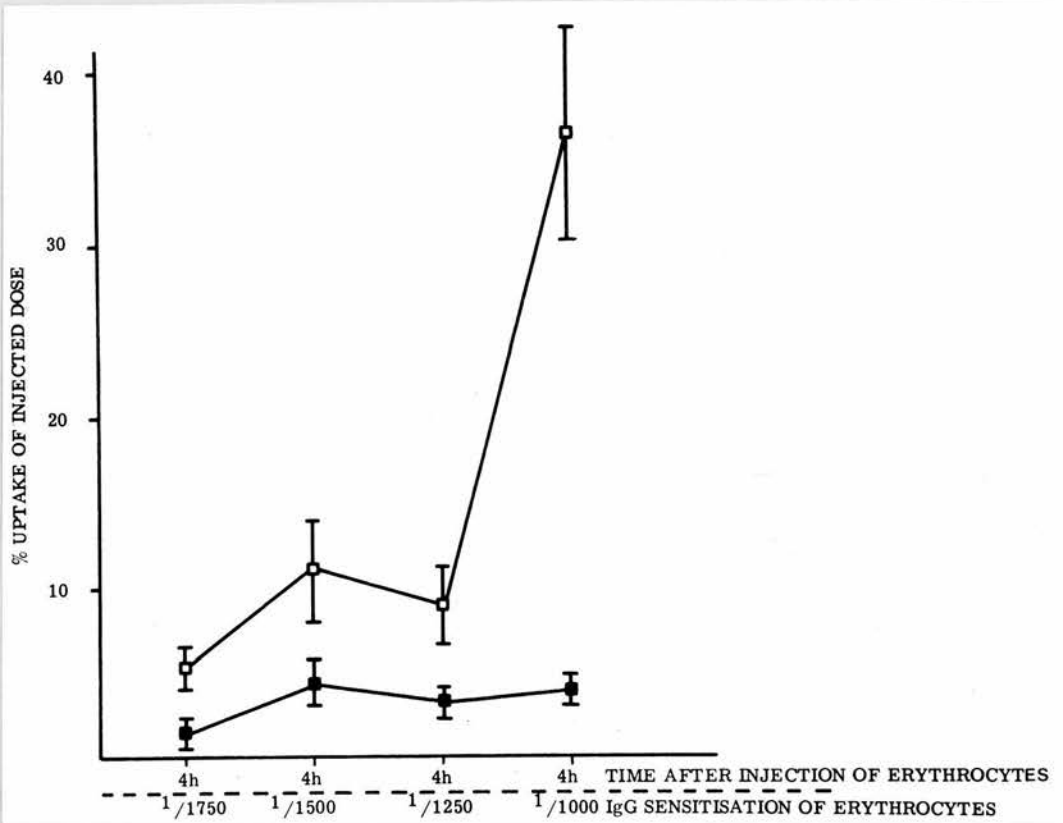


FIGURE XV The effect of increasing erythrocyte IgG sensitisation on hepatic and splenic sequestration patterns. This graph shows the total amount of radioactivity in the whole individual organ. Each symbol represents the mean \pm 2SD values from four normal animals (\square = Liver \blacksquare = Spleen).

b./

b. The effect of cortisone acetate on organ sequestration of 1/1500 sensitised erythrocytes

The only feature of note was an initial depression of sequestration by both spleen and liver at four hours. This was a clearcut effect only in the spleen (Fig. XVI).

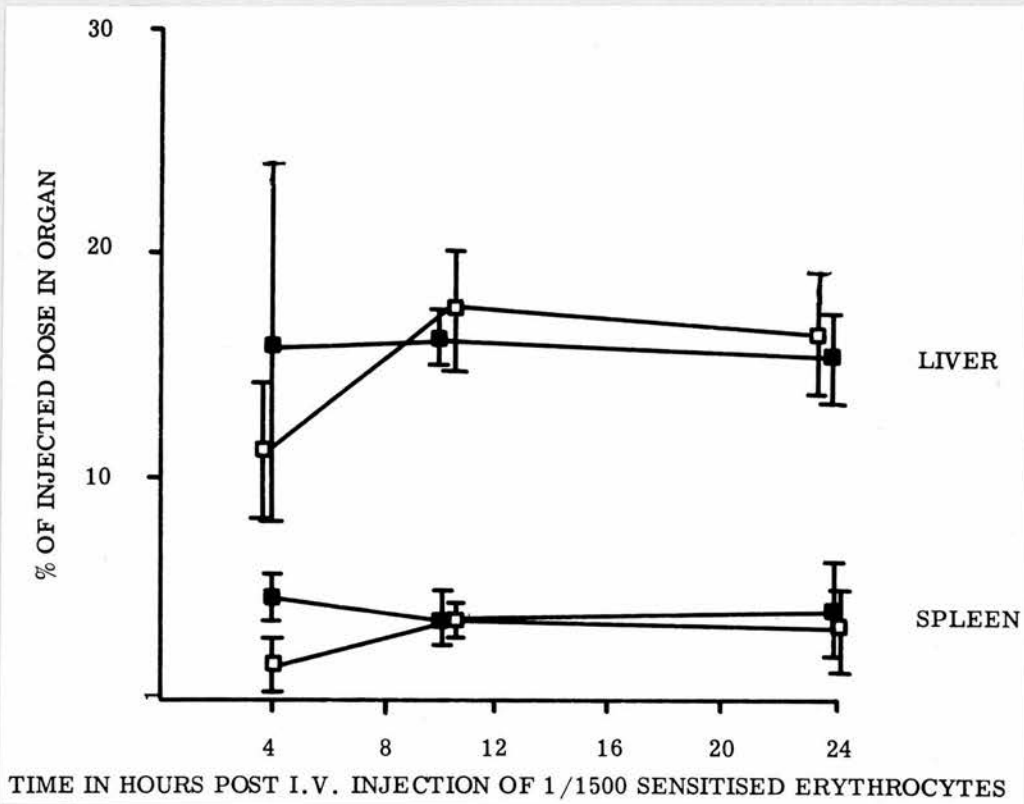


FIGURE XVI The effect of cortisone acetate on hepatic and splenic sequestration of erythrocytes sensitised at 1/1500. Each symbol represents the mean - 2SD values from four mice (□ = cortisone treated mice ■ = normal mice).

5. The effects of cortisone acetate on the splenic and hepatic sequestration of normal and IgG sensitised erythrocytes, expressed as the percentage of the injected dose per unit weight of organ

The results in section 4 were the total organ's (liver or spleen) contribution to erythrocyte clearance from the blood. This section expresses the results per unit weight of organ, thus giving the 'efficiency of sequestration' by spleen and liver.

Expressed in this way the spleen is much more efficient, weight for weight, than the liver at clearing sensitised erythrocytes (Fig. XVII).

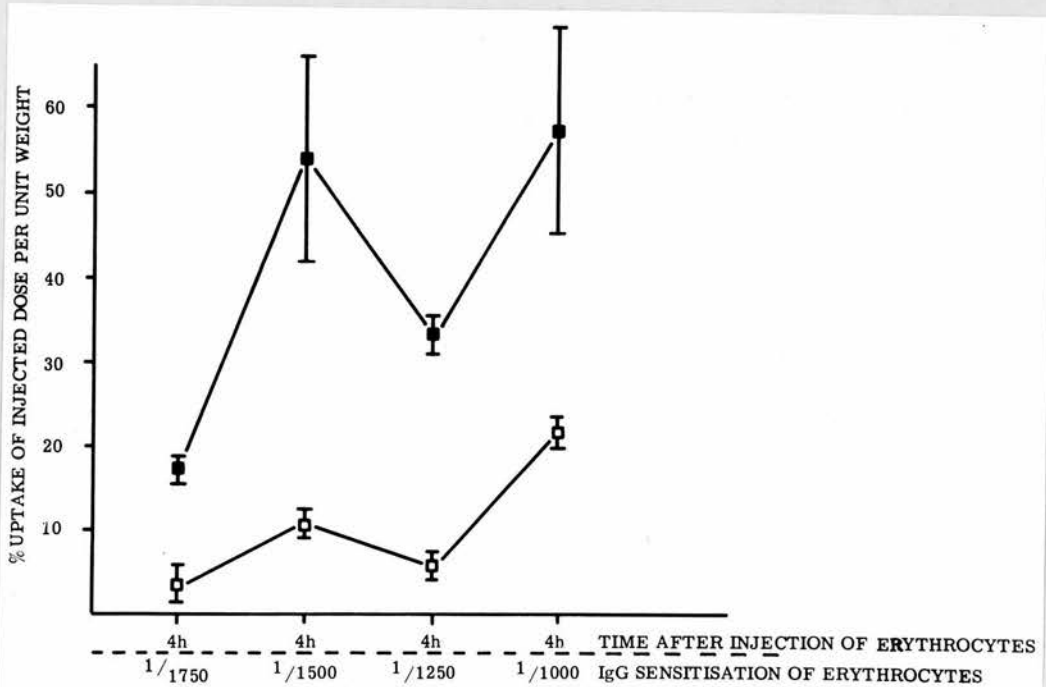


FIGURE XVII The effect of increasing erythrocyte IgG sensitisation on hepatic and splenic sequestration patterns, expressed as scintillation counts per unit weight of organ. Each symbol represents the mean - 2SD values from four mice (■ = spleen □ = liver).

Further the cortisone treated spleen appears to be more efficient, weight for weight, at sequestering erythrocytes, especially those heavily sensitised, (Fig. XVIII) than the untreated spleen.

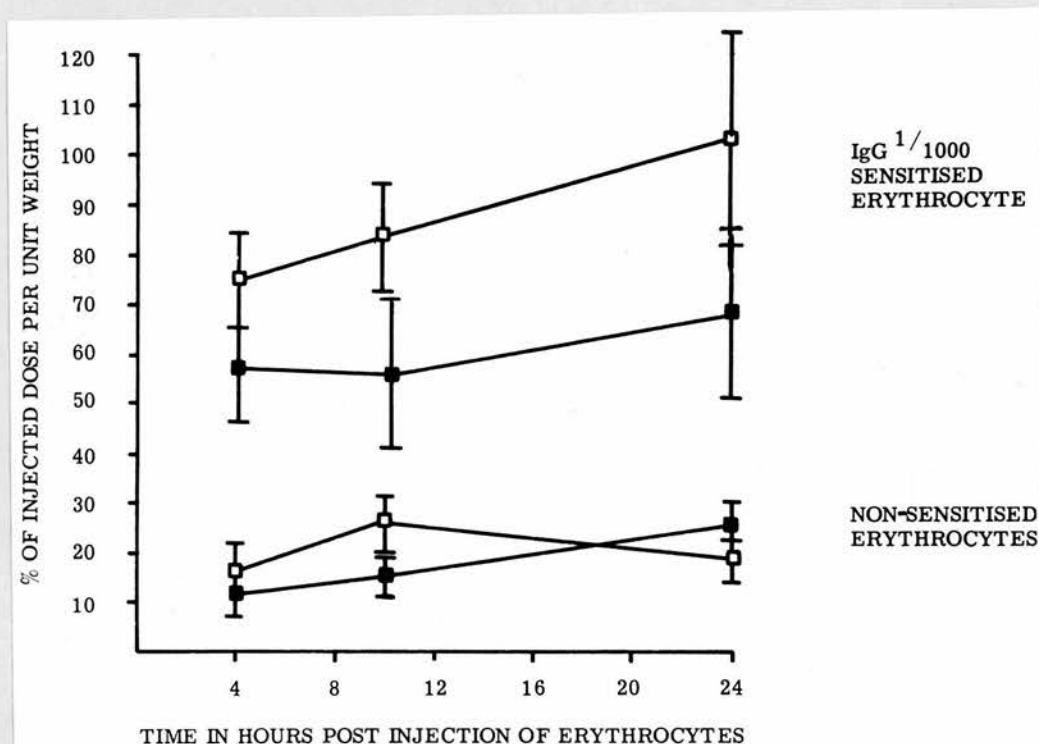


FIGURE XVIII The effect of cortisone acetate on the clearance of 1/1000 IgG sensitised and normal erythrocytes on the mouse spleen. Each symbol represents the mean \pm 2SD on four individual mice. (\square = cortisone treated spleen. \blacksquare = normal spleen).

The liver, following cortisone treatment, is less efficient at clearing 1/1000 sensitised erythrocytes, though unaffected in its clearance of normal red cells (Fig. XIX).

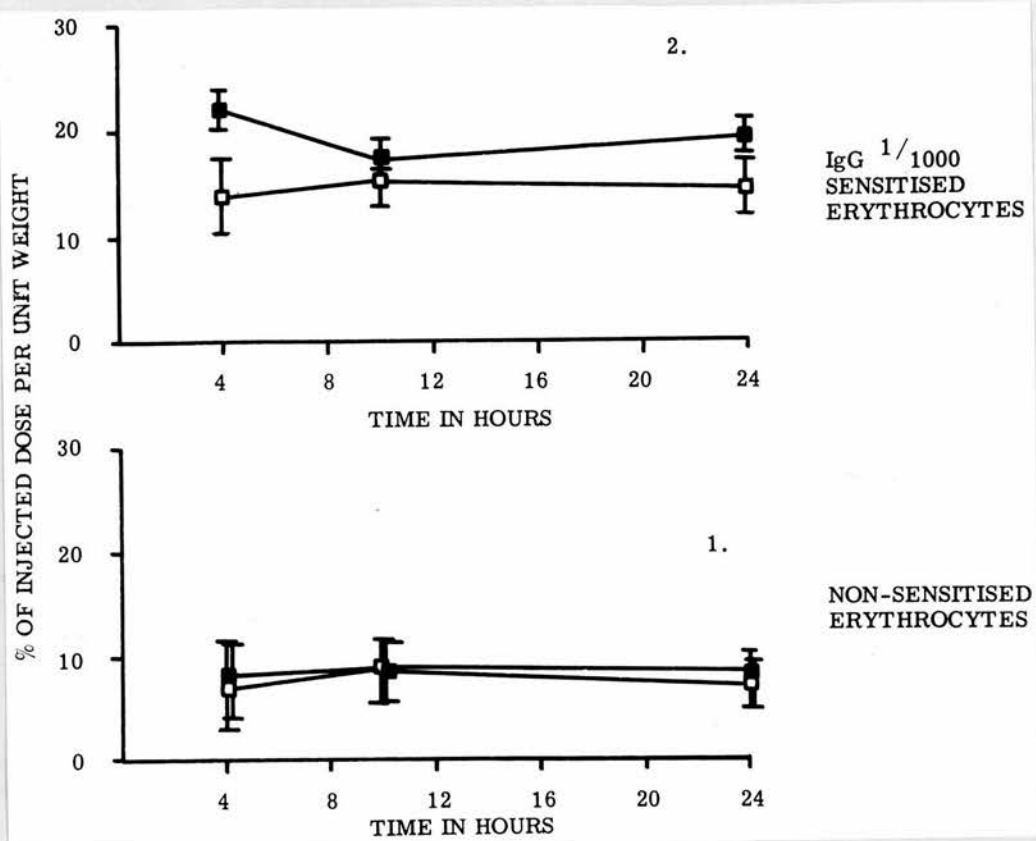


FIGURE XIX The effect of cortisone acetate, on the clearance of 1/1000 IgG sensitised and normal erythrocytes, on the mouse liver. Each symbol represents the mean \pm 2SD on four individual mice (□ = cortisone treated liver. ■ = normal liver).

DISCUSSION

1. The effect of cortisone acetate on murine lymphocyte subpopulations.
 - a. Thymus
 - b. Spleen
 - c. Lymph node

2. The effect of cortisone acetate on murine macrophages.

3. Blood clearance and organ sequestration studies.

4. Conclusions.

Discussion

The main objectives of the experiments described in this chapter were threefold. Firstly, it was wished to define the changes in lymphocyte subpopulations following therapy with cortisone acetate in the mouse thymus, lymph nodes and spleen. Secondly, it was hoped to supplement this information with a study of the in vivo and in vitro effects of steroid on murine peritoneal macrophages. Finally, it was hoped that the blood clearance of IgG sensitised erythrocytes, their subsequent liver and splenic localisation, and any change brought about by steroid therapy could be discussed in terms of subpopulation changes and demonstrated effects on macrophages.

These three topics will now be discussed separately:-

1. The effect of cortisone acetate on mouse lymphocyte subpopulations

a. Thymus

The most definitive effect of cortisone acetate was exerted on the thymus, where the total cell number was reduced to 2% of its original value. Rapid regeneration of the thymic cell population occurred at ten days. Similar results have been reported by other workers (Blomgren and Andersson, 1970). Such rapid repopulation is not unexpected, as the cell turnover of thymic lymphocytes has been reported/

reported to be about 3 - 4 days (Matsuyama et al., 1966; Ernström and Sandberg, 1970) and are amongst the most rapidly dividing cells in the body (Metcalf and Wiadrowski, 1966). Migration from the thymus contributes very little to the turnover, and that which does occur is a late event in the maturational cycle of the thymocyte, involving migration from the medullary sites (Weissman, 1973).

Also demonstrated was a significant fall in the proportion of cells carrying the Θ -antigen. This finding agrees with, and confirms the observation that steroid resistant thymocytes have minimal Θ representation (Takiguchi et al., 1971; Elliott et al., 1971), in contrast to cortisone sensitive cortical thymocytes which bear a high density of Θ determinants (Stobo and Paul, 1973).

Such steroid resistant thymocytes in the medulla have been shown by other workers, to be more effective in inducing graft versus host disease (Blomgren and Andersson, 1969), to be more reactive in mixed lymphocyte culture (Altman and Cohen, 1975) and to show an increased response to P.H.A. (Blomgren and Svedmyr, 1971) when compared with steroid sensitive thymocytes. Despite behavioural similarities, cells situated in the medulla of the thymus should not be equated with peripheral T cells (Elliott et al., 1971; Elliott, 1973), though some lymphocytes leaving the thymus/

thymus for the first time are equipped to participate in peripheral immune reactions (Ritter, 1971).

Though cortisone resistant lymphocytes have a higher capacity for migrating to lymph nodes than to spleen (Lance and Cooper, 1970; Jacobsson and Blomgren, 1972), the spleen cell number, in the experiments in this thesis, regenerated more rapidly than the cell population of the lymph nodes.

The explanation for this discrepancy may be due to two factors, firstly, the recovery of spleen seeking lymphocytes following corticosteroid therapy is more rapid than lymph node seeking cells (Lance and Cooper, 1970; Jacobsson and Blomgren, 1972). Secondly, regeneration might depend upon cell division within the organ itself, the rate of which might be different in lymph node and spleen.

No B lymphocytes were identified in the thymic cell suspensions, in contrast to the findings of Lamelin et al. (1972), who found that the thymic cell suspension always contained a small percentage of B lymphocytes.

b. Spleen

Spleen cell numbers were reduced maximally to 18% of the mean control value. This accords with the findings of other workers (Blomgren and Andersson, 1970; Levine and Claman, 1970). Variation in steroid effect with differing age of mice/

mice has been reported (Altman and Cohen, 1975), the effect being more pronounced, the younger the mouse. At five weeks of age a 15% reduction, and at three months only a 40% reduction of the cell population in the spleen was seen, following steroid therapy. All of the experiments reported here were performed on mice between 1 and 2 months of age.

In the spleen, both T and B-lymphocytes were reduced in absolute terms. B lymphocytes were shown to be relatively more sensitive by means of the T/B lymphocyte ratio, which rose to a maximum of 2.65 at five days. This differential effect was more clearly demonstrated, when an experiment, in which cortisone acetate was given over a longer period, was performed.

There is some controversy as regards the type of murine lymphocyte most affected by steroid, and also on the functional integrity of the remaining cells. Data supporting selective sensitivity of B-lymphocytes has been presented by Cohen and Claman (1971) and Lane and Raff (quoted in Greaves et al., 1974a). Three groups of workers (Segal et al., 1972; Vann, 1974; Lee et al., 1975), maintain in contrast, that immunocompetent B-lymphocytes are not selectively sensitive to cortisone and that cortisone induces functional defects in T-helper cells.

A/

A recent review on the effect of steroid on T lymphocytes (Bach et al., 1975) concludes, that in general, quiescent T-lymphocytes are resistant, whereas T-lymphocytes which have undergone activation or are proliferating, are sensitive to such treatment.

The majority of the experiments quoted above have not depended on direct quantitation of B-lymphocytes, but on assay by functional aspects (e.g., antibody formation) as an indicator of the number of B-cells. The only similar experiment in the literature to the work reported in this thesis, is that of Veldkamp et al. (1974), who reported that three days following cortisone therapy, there was a relative increase in T-cells, due to a reduction in 'null cells', without an absolute reduction in B-cells.

The percentage of cells without surface Θ or immunoglobulin ('null cells') was high (35%) in their experiments, and Raff (1970) also reported high levels of such 'null cells' (24%) in spleen suspensions. 'Null cells' in the experiments in this thesis were 41% in the controls. These figures contrast with those of Stobo et al. (1973) who found 3-14% of 'null cells' in mouse spleen cell suspensions, and only 5% in C3H mice, using fluorescent techniques.

The/

The only difference in technique in this thesis, was that homogenates of tissue were used, whereas Stobo et al. (1973) gently teased the spleens to release the cells, and all the fluorescent labelling procedures were carried out at 4°C. Possibilities that help to 'close the gap' are that the cell suspensions used in the experiments in this thesis contained a significant percentage (10%) of macrophages, which would not label with fluorescent antisera. Further, the incubation at room temperature with the fluorescent anti-mouse immunoglobulin caused capping of the Θ -antigen. Pinocytosis, which is likely to have occurred, would make some T-lymphocytes, which had a low starting surface density of Θ , in fact appear negative. Certainly the level of 'null cells' in my experiments, are more in keeping with those of Raff (1970) whose technique was used in this thesis.

'Null cells', in particular those described by Stobo et al. (1973), are thought to be immature B-lymphocyte precursors (Greaves et al., 1974a). Though Veldkamp et al. (1974) found that the relative rise in T-cells was due to a loss of non-reactive cells, in the experiments reported here, there was no evidence of a differential sensitivity of 'null cells' compared to T-lymphocytes.

c./

c. Lymph Node

The lymph node results showed a fall in cell number, to 12-15% of the mean control level. Repopulation of these organs was slower than thymus and spleen, and even at the end of the observations had not returned to normal. The B-lymphocytes in the lymph nodes showed a similar increased sensitivity to steroids when compared to T-lymphocytes, the rise in the T/B lymphocyte ratio persisting up to the end of the experiment. As the total cell number had by this time increased, but had not returned completely to normal, these findings suggest that lymph node regeneration following steroid therapy is initially by T-lymphocytes.

2. The effect of cortisone acetate on murine macrophages

Turning now to the steroid effect on macrophages, it was found that steroids, if used in a pharmacological dose, exerted an inhibitory effect on the phagocytosis of IgG sensitised erythrocytes by monolayers of mouse peritoneal macrophages. The amount of cortisone required to produce this effect was high. Other features of the cultures with the high dose cortisone acetate, were a reduced density of the monolayer and the presence of increased numbers of bizarre macrophages, this latter feature was not further investigated./

investigated.

In vivo cortisone acetate did not affect the phagocytic ability of subsequently cultured peritoneal macrophages, but the addition of cortisone in vitro, to cultures from previously injected mice, was inhibitory to phagocytosis.

There is general agreement that small doses of cortisone stimulate phagocytic activity in the reticuloendothelial system of rats, mice and guinea pigs, whereas large doses induce blockade (Nelson, 1969). The results in this thesis would support this contention, certainly on in vitro experiments. Other observations which support the anti-phagocytic effect that can be produced by steroids, are the observations by Lee et al. (1975) who found a reduction in the frequency of macrophages full of endocytosed erythrocytes in cortisone treated cultures, and Weiner et al. (1967) who showed, using electron microscopy, that cells in cortisone treated rats failed to phagocytose carbon particles.

In contrast, North (1972) maintains, that although there is a failure of monocyte derived macrophages to accumulate in adequate numbers at sites of bacterial implantation, that the functional potential of the macrophage system in cortisone treated mice remains essentially intact. In the human recently, Rinehart et al. (1975) were unable to demonstrate impaired/

impaired phagocytic ability following in vivo treatment with cortisone, though there was an impairment of monocytic bactericidal and fungicidal activity. They also quote in vitro experiments which showed a similar trend. The dose of cortisone used in these experiments was not high.

The negative results of the effect of in vivo steroids could be explained by reversal to normal on removal to the in vitro test. Certainly, there is much indirect evidence (to be discussed) that steroids in vivo can affect monocyte phagocytic function. Whether this is directly, by a reduction in number of receptor sites or interference with membrane integrity, or due to mechanisms such as the reduced production of macrophage inhibition factor by lymphocytes, demonstrated by Rosenthal and Balow (1975), requires further investigation.

3. Blood Clearance and Organ Sequestration Studies

Demonstrated in this series of experiments was an increased rate of clearance of IgG sensitised red cells when compared to non-sensitised erythrocytes. Cells sensitised at a level of 1/1250 were cleared more rapidly than the other two levels of sensitisation (1/1500 and 1/1750). The latter were removed at similar rates over the periods of observation. The demonstrated divergence between the/

the two groups was not great. Perhaps the lack of accuracy of the mode of sensitisation of the red cell with antibody contributed to this result. Other authors, using sensitive techniques to monitor antibody amount on the erythrocyte, have clearly shown a fall of clearance rate with reduced red cell sensitisation. The latter experiments were carried out on a relatively steroid resistant animal, guinea pigs (Atkinson et al., 1973).

Also demonstrated was a reversal towards normal of the clearance rates of 1/1500 sensitised erythrocytes by cortisone therapy. No definitive answer can be given to the mode of action of steroids in this respect but some suggestions can be made. Kaplan and Jandl (1961) have presented evidence to show that the inhibition of sequestration of sensitised erythrocytes in rats, by cortisone therapy, is not by a specific action of steroids on immune processes. They suggest that haemodynamic changes in the liver and spleen could explain the inhibition of clearance they demonstrated.

It is conceivable that in the mice studied here, structural differences, caused by the rapid disappearance of up to 80% of the nucleated cells in the spleen, could influence the clearance of red cells in this organ.

With/

With regard to sequestration, the spleen is more efficient than the liver (per unit weight). The increased efficiency of sequestration shown by spleens from cortisone acetate treated mice, indicates that the cells responsible for phagocytosis are relatively resistant to such treatment, when compared with lymphocytes.

The liver, in contrast, following cortisone acetate therapy, showed a reduced efficiency of sequestration most clearly demonstrated with heavily sensitised erythrocytes. The lack of weight loss of the liver following such treatment, in conjunction with the evidence in the literature that monocytes are resistant to steroid lysis (Thomson and Van Furth, 1970), favours a qualitative rather than a quantitative effect of steroids on mononuclear phagocyte function.

Other authors have postulated that steroids may act by interfering with the production, or the efficiency, of macrophage membrane receptors (Atkinson and Frank, 1974).

4. Conclusions and suggestions for further experiments

In conclusion, the experiments in this chapter have shown that in mice, steroid therapy reduces the number of lymphocytes in thymus, spleen and lymph nodes. In spleen and lymph nodes B lymphocytes were demonstrated/

demonstrated to be more sensitive than T-lymphocytes to cortisone acetate. The thymic remnant, following such therapy, had a low percentage of cells with surface Θ antigen.

Despite a reduction to 18% of the mean control cell number in the spleen with steroids, such treatment resulted in an organ which was more efficient, in terms of unit weight, at sequestering red cells. In contrast, there was a reduction of efficiency of clearance by the liver without a corresponding fall in the weight of this organ. The evidence is not inconsistent with a qualitative effect on mononuclear phagocyte function. The molecular mechanism responsible for such an effect requires elucidation, but I have demonstrated clearly, that cortisone acetate in high dosage in vitro has an inhibitory effect on phagocytosis by such cells.

Further experiments should include an accurate enumeration of macrophage number in liver and spleen with such treatment. Investigation of the membrane effects of steroids on mononuclear phagocytes is more difficult. It would be interesting to find whether steroid therapy prevented the formation of activated macrophages, especially as the latter carry twice as many Fc receptors as unactivated cells (Arend and Mannik, 1973)./

1973). Further the kinetics of regeneration of Fc receptors, following exposure to steroids could be studied, using experiments similar to those of Schmidt and Douglas (1972). These authors showed that the uptake of sensitised erythrocytes by macrophages was reduced by 90%, after preloading the monolayer with latex particles. It took six hours for the receptor activity to return.

Regardless of the mechanisms involved, the administration of corticosteroids decreases the clearance and sequestration of IgG sensitised erythrocytes. This effect may be important in the induction of remission in patients with warm antibody haemolytic anaemia, and may provide an explanation for the finding of patients in remission, with a persistently positive antiglobulin test.

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