IMMUNE COMPLEX DETECTION BY IMMUNOFLUORESCENCE ON POLYMORPHONUCLEAR LEUCOCYTES

AN EXPERIMENTAL AND CLINICOPATHOLOGICAL STUDY

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE GENEESKUNDE
AAN DE ERASMUS UNIVERSITEIT TE ROTTERDAM
OP GEZAG VAN DE RECTOR MAGNIFICUS
PROF. DR. B. LEIJNSE
EN VOLGENS BESLUIT VAN HET COLLEGE VAN DEKANEN.
DE OPENBARE VERDEDIGING ZAL PLAATS VINDEN OP
WOENSDAG 1 JUNI 1977 DES NAMIDDAGS
TE 4.15 UUR PRECIES

DOOR

Jan Willem Steffelaar

GEBOREN TE MIDDELBURG

1977
BRONDER-OFFSET B.V. – ROTTERDAM

PROMOTOR : PROF. DR. R.O. v.d. HEUL
CO-REFERENTEN : MEVR. DR. TH.M. FELTKAMP-VROOM
PROF. DR. M. FRENKEL

Publication of this thesis was partly financed by a grant of the Dutch National Kidney Foundation ("Nier Stichting Nederland")

CONTENTS

LIST OF ABBREVIATIONS

AC-activity	= anticomplementary activity
AcH	= acute hepatitis
ANA	= antinuclear antibodies
BSA	= bovine serum albumin
C	= complement
CAH	= chronic active (aggressive) hepatitis
CPH	= chronic persistent hepatitis
Cirrh.	= cirrhosis
DNA	= desoxy ribonucleic acid
EDTA	= ethylene diamine tetraacetate
FITC	= fluoresceine isothiocyanate
GBM	= glomerular basement membrane
GN	= glomerulonephritis
HBcAg	= hepatitis B core antigen
HBsAg	= hepatitis B surface antigen
anti HBc	= anti HBcAg immunoglobulin
anti HBs	= anti HBsAg immunoglobulin
IF(T)	= immunofluorescence (technique)
Ig	= immunoglobulin
MIAA	= monoiodine acetic acid
PAN	= polyarteritis nodosa
PBS	= phosphate buffered saline
PEG	= polyethylene glycol
PMN	= polymorphonuclear leucocytes
SLE	= systemic lupus erythematosus

CHAPTER I : INTRODUCTION

Diseases, which are the result of immune reactions associated with tissue damage may be caused by several mechanisms. The immunologic mechanisms resulting in tissue damage have been categorized in 4 types (Gell and Coombs, 1968).

1. the anaphylactic type, mediated by IgE type of antibodies,resulting in immediate release of vasoactive amines (e.g. histamine).
2. the cytotoxic type, mediated by IgG and IgM type of antibodies and resulting in phagocytosis of the target cells or lysis by complement activation.
3. the immune complex type, with either primary localisation of the immune complexes in the tissues (Arthus reaction) or circulating immune complexes with secondary tissue localisation.
4. the delayed type or cellular immune response mediated by thymus derived T-lymphocytes.

In the studies to be reported, only diseases caused by the type 3 reaction, mediated by circulating immune complexes, are of concern. Examples of human diseases caused by the immune complex mediated type of tissue damage are a.o. serum sickness, systemic lupus erythematosus (SLE) and several types of glomerulonephritis.

The source of the antigens involved may be exogenous or endogenous; when endogenous, autoimmune diseases will be the result of such immune reactions.

To monitor the activity of the immune complex mediated type of disease, it is of importance to dispose of techniques which are able to detect immune complexes in the circulation.

In order to be of clinical interest, these techniques should meet the following requirements:

1. The results of the test should correlate with clinical signs and symptoms of immune complex mediated disease,

i.e. the test should discriminate potentially pathogenic from "harmless" complexes.
2. The test should include the possibility to detect the antigen involved in immune complex formation.
3. Preferentially, the laboratory outfit needed should be relatively simple, and to perform the test, only commercially available reagents should be necessary. An immune complex detection technique, which meets all these requirements, is not available.

Tests to measure antigen-antibody interactions have been put into three categories by Minden and Farr (1971):
1. Tests based on the primary interaction between antigen and antibody.
2. Secondary tests, which measure in vitro manifestations that may occur following primary interactions.
3. Tertiary tests, based on in vivo manifestations of primary antigen-antibody interactions.

These tests may be designed for the detection and quantitation of known antigens or specific antibodies on one hand, on the other hand some techniques are designed for the detection of antigen-antibody complexes.

Primary binding tests, designed for the quantitative measurement of antigen-antibody complexes are based on changes in physicochemical properties of antigens and antibodies after immune complex formation and include fractionated precipitation with ammonium sulphate (Farr, 1958), analytical ultracentrifugation and gelfiltration techniques.

Several secondary tests, which have been used for the detection of immune complexes are based on their interaction with the complement system. Since Agnello et al. (1970) described the agar gel precipitation technique with C1q globulin for demonstrating immune complexes, radiolabeled C1q has been used in combination with precipitation by polyethylene glycol (Nydegger et al. 1974) and in a test based on the inhibition by immune

complexes of the C1q uptake by sensitized erythrocytes (Sobel et al., 1975).

An example of a tertiary test for the detection of immune complexes is the intradermal injection of serum in the skin of guinea pigs and to measure the immediate local inflammatory response (McAuliffe et al., 1973).

The fate of circulating immune complexes is dependent on the type of the antigen, the antigen/antibody ratio and the immunoglobulin class and subclass of the antibody involved. Most antigen-antibody complexes are rapidly cleared from the circulation by phagocytosis by the reticulo endothelial system, especially by the Kupffer cells in the liver (Benacerraf et al. 1959). On the other hand, the immune complexes may be deposited in renal glomeruli, vessel walls and other tissues, as has been confirmed in many studies of experimental and human disease since the classical experiments on the pathogenesis of serum sickness by Dixon et al. (1958).

Phagocytosis of Ig and C by polymorphonuclear leucocytes (PMN) has been demonstrated experimentally in vivo (Cochrane et al., 1959) and in vitro (Parker and Schmid, 1962) and has also been observed in synovial fluid and peripheral blood from patients with rheumatoid arthritis and SLE (Rawson et al., 1965; Vaughan et al., 1968; Cats et al., 1975). In addition, Cats et al., (1975) reported in vitro phagocytosis by normal donor PMN of Ig and C from the sera of patients with rheumatoid arthritis and SLE.

Therefore, evidence of phagocytized Ig and C in peripheral blood PMN might be an important parameter in monitoring diseases caused by circulating immune complexes.

In this thesis some experiments and clinical, histological and IF studies are reported, which were designed with the purpose to develop a technique which enables to monitor diseases, mediated by circulating immune complexes. This technique is based on phagocytosis of

immune complexes by PMN, both in vivo and in vitro. The studies included patients with SLE and hepatitis B, since SLE is one of the classical examples of an immune complex mediated disease (Cochrane and Koffler, 1973), and systemic manifestations in hepatitis B (e.g. arthritis, polyarteritis nodosa and glomerulonephritis) are also attributed to circulating immune complexes, presumably of HBsAg and anti HBs (Kohler, 1973; Gocke, 1975). In addition, the influence of the antigen-antibody ratio on the in vitro phagocytosis of artificially prepared HBsAg-anti HBs immune complexes was studied.

CHAPTER II

In this chapter a technique is described for the isolation and IF-study of PMN, which facilitates the screening of large numbers of PMN obtained from relatively small volumes of blood. In addition, the results of IF-studies of PMN from the blood of nine patients with SLE in an active stage of the disease are reported. These results are compared with those obtained from matched normal controls and from seven patients with high levels of paraproteins.

Clin. exp. Immunol. (1976) **23**, 272–278.

Immune complex detection by immunofluorescence on peripheral blood polymorphonuclear leucocytes

J. W. STEFFELAAR, CLAIRE B. DE GRAAFF-REITSMA & THEA M. FELTKAMP-VROOM *Department of Immunohistopathology of the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service and Laboratory of Experimental and Clinical Immunology, University of Amsterdam and Department of Clinical Pathology of the Medical Faculty of the Erasmus University, Rotterdam*

(*Received* 21 *May* 1975)

SUMMARY

Peripheral blood polymorphonuclear leucocytes (PMN) from patients with SLE in active stage were isolated and frozen in gelatin capsules. Sections cut from these frozen cell preparations were examined for the presence of inclusion bodies using the immunofluorescence technique. It was demonstrated that up to 80% of the polymorphonuclear cells contain small granular inclusions when tested with anti-IgG, -IgM, -IgA and antisera specific for several C components. Incidentally large homogeneous inclusions similar to the *in vitro* LE cell phenomenon were observed. PMN from normal controls matched for age, sex and race showed no inclusion bodies. Three out of six patients with paraproteinaemia of different Ig class had PMN inclusions consisting of the corresponding paraprotein only. These data suggest that inclusions in PMN from SLE patients are phagocytozed immune complexes.

INTRODUCTION

Since Farr in 1958 introduced fractionated globulin precipitation with ammonium sulphate for the detection of circulating immune complexes, many other techniques have been developed for this purpose, e.g. analytical ultracentrifugation, agar gel precipitation with C1q globulin and histamine release (reviewed by Cochrane & Koffler, 1973). As it is known that antigen–antibody complexes are eliminated from the circulation by phagocytosis effected by the reticuloendothelial system (RES) and by deposition in renal glomeruli and other tissues (Benacerraf, Sebestijen & Cooper, 1959; Dixon *et al.*, 1958, Orozco, Jasin & Ziff, 1970), the investigation of biopsy specimens of kidney and other tissues by means of immunofluorescence might also provide evidence for the presence of circulating immune complexes. For obvious reasons, however, frequent taking of biopsy specimens from patients is not admissible, which makes this method less suitable for follow-up studies of immune complex disease in man.

Phagocytosis of immune complexes by peripheral blood polymorphonuclear leucocytes (PMN) has been demonstrated experimentally *in vivo* (Cochrane, Weigle & Dixon, 1959) and *in vitro* (Parker & Schmid, 1962); the phenomenon has also been observed in rheumatoid synovia fluids (Rawson, Abelson & Hollander, 1965). Therefore, it is likely that PMN also

Correspondence: J. W. Steffelaar, Department of Clinical Pathology, Medical Faculty of the Erasmus University, P.O. Box 1738, Rotterdam, the Netherlands.

contribute to the elimination of circulating antigen–antibody complexes. Although immunoglobulin (Ig) inclusions in PMN have been noted in several diseases (Vaughan *et al.*, 1968), the possible significance of this phenomenon as a parameter for the presence of circulating immune complexes has as yet not been studied. The purpose of the present study was to investigate, whether evidence for immune complex phagocytosis by PMN could be obtained, using the immunofluorescence technique. Since current knowledge points strongly towards circulating immune complexes as a pathogenetic factor in SLE (reviewed by Cochrane & Koffler, 1973), PMN from nine SLE patients in an active stage of the disease were studied. The results were correlated with those obtained by light microscopic and immunofluorescence studies of biopsy specimens from the same patients.

MATERIALS AND METHODS

Subjects. The study comprised nine patients with SLE, as diagnosed according to the criteria proposed by the American Rheumatism Association (Cohen *et al.*, 1971). All patients were in an active stage of the disease at the moment of blood sampling. From eight out of these nine patients renal, liver or skin biopsy specimens were available for examination with light microscopy and from six out of these eight patients such biopsy specimens were also available for immunofluorescence studies.

Normal controls consisted of nine race-, age- and sex-matched individuals with no personal history of allergic disorders or recent bacterial and viral infections. In addition, seven patients with hypergammaglobulinaemia, unrelated to SLE were included as controls. Three of these patients had an IgG paraproteinaemia with an IgG level of 250, 280 and 840 i.u./ml respectively, two had an IgA paraproteinaemia with an IgA level of 3680 and 6840 i.u./ml respectively, one had an IgM paraproteinaemia with a level of 7552 i.u./ml and one an IgE level of 28,000 i.u./ml.

Polymorphonuclear leucocytes. PMN were isolated according to Böyum (1968) with a slight modification: to each of two tubes containing 10 ml of fresh, defibrinated venous blood 2·5 ml of 5% dextran in 0·9% saline (molecular weight 200,000) (Poviet, Amsterdam, the Netherlands) were added. Red blood cells (RBC) were allowed to sediment at 37°C for 30 min. Each supernatant was diluted with one volume of minimal essential medium (MEM) (Gibco, Grand Island, New York) containing 25 mM Tris (pH 7·4) at 20°C and layered on top of 3 ml Ficoll–Isopaque mixture of specific gravity 1·077 present in each of two tubes with a diameter of 14 mm.

After centrifugation at 20°C for 40 min at 400 *g* the interphase, containing mononuclear cells, was carefully aspirated and the medium and Ficoll–Isopaque mixture were discarded. To the pellet 3 ml of an ice-cold isotonic NH_4Cl solution containing 0·155 M NH_4Cl, 10 mM $KHCO_3$ and 0·1 mM EDTA (pH 7·4) were added to resuspend the pellet and to accomplish lysis of contaminating RBC (Roos & Loos, 1970). To achieve this the tubes were kept at 0°C for 5 min under constant stirring. After that the tubes were filled with washing fluid as described by Hijmans, Schuit & Klein (1969) and centrifuged for 10 min at 90 *g*. The pellet was resuspended in ten drops of washing fluid and fixed for 5 min by adding ten drops of a 2% paraformaldehyde solution in phosphate-buffered saline, 0·01 M, pH 7·8 (PBS) as previously described (Smit *et al.*, 1974). After centrifugation for 10 min at 90 *g* the supernatant was removed and the pellet washed twice by resuspension in 10 ml washing fluid and subsequently centrifuged again. The PMN were finally suspended in five to seven drops of 5% gelatin in washing fluid, counted in a Bürker chamber and frozen in a gelatin capsule in liquid nitrogen as described earlier for minute tissue specimens (Feltkamp-Vroom & Boode, 1970). The cellular concentration ranged from 6 to 10×10^6 PMN/ml, contamination with RBC or platelets was negligible. Differential counts generally showed 1–3% mononuclear cells.

Immunofluorescence procedure for PMN. The indirect immunofluorescence technique was used for the demonstration of IgG, IgG1, IgG2, IgG3, IgG4, IgM, IgA, Clq, C3B, C3c, C3d and C4. Cryostat sections of 4 μm were cut from the frozen gelatin capsules, airdried for 30 min and washed for 30 min with PBS. Then the sections were incubated for 30 min at room temperature with the specific rabbit anti-human antisera. All antisera were prepared in the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service and tested for specificity by immunoelectrophoresis, gel double diffusion and the tanned red cell haemagglutination technique. As second layer horse anti-rabbit immunoglobulin serum conjugated to fluorescein-isothiocyanate (FITC) (The & Feltkamp, 1970), was used. The presence of IgE was studied in the direct technique using a FITC labelled anti-polyclonal IgE serum, as described previously (Feltkamp-Vroom *et al.*, 1975). Sections incubated with normal rabbit serum as first layer as well as sections incubated with the conjugated horse anti-rabbit serum only were used as negative controls.

Nuclear counterstaining was performed by incubating the sections with ethidium bromide (0·02 mg/ml in PBS, Calbiochem, Santiago, California) for 2 min (Udenfriend, 1969).

of Ig, and C components in the glomerular capillary walls except for that of patient 7 (Table 1), which demonstrated slight proliferative glomerular changes only. The one liver biopsy specimen available (patient 5, Table 1), showed massive cytoplasmic fluorescence of Kupffer cells for IgG, IgM, IgA and C components. None of the tissues showed IgE in granular deposits or Kupffer cells.

DISCUSSION

Freezing of PMN suspensions in gelatin capsules and subsequent cutting of 4 μm sections in a cryostat proved to be a useful technique in order to obtain preparations that are suitable for immunofluorescence studies of these cells. The main advantages of this technique are that firstly a large number of sections can be cut from a rather small sample and that secondly sections can be cut at any desired moment after preparation of the frozen suspensions. By fixation in 1% paraformaldehyde prior to freezing the cells are well preserved, while significant alteration of antigens does not occur (Smit *et al.*, 1974). Nuclear counterstaining with ethidium bromide allows easy identification of the nuclei (Udenfriend, 1969), using the same excitation light as for FITC fluorescence. This quick and simple method has proved to be very useful for determining the number of cells that do not show FITC fluorescence.

With the immunofluorescence procedure described in this paper we could demonstrate that PMN isolated from venous blood of SLE patients contain cytoplasmic inclusions consisting of IgG, IgM and IgA as well as of the complement components C1q, C3c, C3d and C4. It is not surprising that no inclusions with IgE were found, since in a previous study it could be established that IgE cannot be found in immune complex deposits (Feltkamp-Vroom *et al.*, 1975). The failure to demonstrate C3B, the conformation determinant of C3, is just a matter of course since this determinant rapidly disappears after activation of C3 (Molenaar *et al.*, 1974).

This PMN inclusion phenomenon may represent either phagocytized Ig aggregates or immune complexes. Although it is not possible to exclude the presence of Ig aggregates some of our observations are in favour of immune complex phagocytosis. If aggregates of Ig and subsequent phagocytosis occur in SLE patients, our findings do not suggest that this is caused by changes in the concentration of Ig in their serum, since PMN from normal controls with only slightly lower Ig levels did not show inclusions. Moreover, only three out of the six patients with moderately to extremely high serum levels of paraproteins showed PMN with cytoplasmic inclusions, and those who reacted positively did so rather weakly, since the number of PMN with inclusions only positive for the corresponding Ig class of the paraprotein, was very low. Although this finding may be the result of paraprotein aggregation with subsequent phagocytosis, it is not likely that the same mechanism accounts for the large quantities of Ig of several classes together with C ingested by PMN as observed in our SLE patients. Furthermore, the granular deposition of Ig and C components in the glomerular capillary walls of all kidney biopsy specimens available except for one are suggestive for the presence of circulating immune complexes. Moreover, the many Kupffer cells in the one liver biopsy specimen showing massive cytoplasmic fluorescence of Ig and C indicate that also in man Kupffer cells play an important role in the elimination of circulating immune complexes as has been demonstrated in mice, rabbits and monkeys (Benacerraf *et al.*, 1959; Mannik & Arend, 1971).

Many reports have provided data, that are suggestive for DNA–anti-DNA complexes being responsible for glomerular lesions in SLE (Koffler, Agnello & Kunkel, 1974; Koffler, Schur & Kunkel, 1967; Robitaille & Tan, 1973). However, our studies are not suggestive for the presence of DNA in the phagocytozed material seen as small inclusions.

In view of their size and localization these inclusions may be phagolysosomes and may contain deoxyribonuclease (Cohn & Hirsch, 1960). Should this hypothesis be true the

deoxyribonuclease will digest DNA, which makes the detection of DNA, if present as the antigen of immune complexes in SLE, when phagocytozed by PMN of SLE patients, impossible. In the large homogeneous inclusion bodies we, however, were able to demonstrate DNA. Considering the similarity of these inclusions to those found in the LE-cell phenomenon, we suppose that the large homogeneous inclusion bodies consist of phagocytozed nuclear material. The large amount of this material in which DNA has been found, leads probably to its detection as it cannot be broken down in a short lapse of time. Since *in vitro* studies of the catabolism of bovine serum albumin (BSA) and antibody by rabbit PMN after phagocytosis of BSA–anti-BSA complexes have demonstrated that up to 46% of the amount of antigen that was originally present was broken down within 24 hr (Cochrane *et al.*, 1959) and since *in vivo* studies on circulating PMN indicate that the whole population is replaced about two and a half times within 24 hr (Boggs, 1967), the observation of PMN with immunoglobulin and C inclusions, points to a very active and continuous phagocytosis in SLE patients. Therefore, immunofluorescence studies on PMN might provide an important parameter for the determination of the activity of the disease and of the effect of therapy. In follow-up studies PMN inclusions will have to be studied in relation to the presence of circulating immune complexes as demonstrated by direct methods, as well as in relation to serological findings and clinical parameters. The impaired *in vitro* phagocytosis by PMN from SLE patients, which has been reported (Brandt & Hedberg, 1969; Oroczo *et al.*, 1970), is another point that remains to be elucidated. Our findings suggest that this impairment is due to phagocytosis by PMN prior to the *in vitro* test.

ADDENDUM

Following submission of this manuscript it was found that similar studies on granulocytes isolated from heparinized blood of SLE patients contain less and smaller granules than those isolated from defibrinated blood. Therefore it is likely that during the defibrination procedure with glass beads granulocytes still phagocytoze immune complexes and nuclear material bound to ANF.

The authors are grateful to Dr Marijke van der Giessen and co-workers for the production of the antisera, to Dr C. P. E. Engelfriet and co-workers for the specificity testing of the antisera by haemagglutination, to Dr Eveliene E. Reerink-Brongers and co-workers for the Ig determinations, Mrs Ellen Hertroijs and Mrs Irma Moerkerken-Mersel for secretarial help and Mrs Anneke Knappers for assistance in preparing the manuscript.

REFERENCES

BENACERRAF, B. SEBASTIJEN, M. & COOPER, N.S. (1959) The clearance of antigen–antibody complexes from the blood by the reticuloendothelial system. *J. Immunol.* **82,** 131.

BOGGS, D.R. (1967) The kinetics of neutrophilic leukocytes in health and in disease. *Semin. Haemat.* **4,** 359.

BÖYUM, A. (1968) Isolation of mononuclear cells and granulocytes from human blood. *Scand. J. clin. Lab. Invest. Supplement* 97.

BRANDT, L. & HEDBERG, H. (1969) Impaired phagocytosis by peripheral blood granulocytes in systemic lupus erythematosus. *Scand. J. Haemat.* **6,** 348.

COCHRANE, C.G. & KOFFLER, D. (1973) Immune complex disease in experimental animals and man. *Advanc. Immunol.* **16,** 185.

COCHRANE, C.G., WEIGLE, W.O. & DIXON, F.J. (1959) The role of polymorphonuclear leukocytes in the initiation and cessation of the Arthus vasculitis. *J. exp. Med.* **110,** 481.

COHEN, A.S., REYNOLDS, W.E., FRANKLIN, E.C., KULKA, J.P., ROPES, M.W., SCHULMAN, L.E. & WALLACE, S.L. (1971) Preliminary criteria for the classification of systemic lupus erythematosus. *Bull. rheum. Dis.* **21,** 643.

COHN, Z.A. & HIRSCH, J.G. (1960) The isolation and properties of the specific cytoplasmic granules of rabbit polymorphonuclear leukocytes. *J. exp. Med.* **112,** 983.

DIXON, F.J., VAZQUEZ, J.J., WEIGLE, W.O. & COCHRANE, C.G. (1958) The pathogenesis of serum sickness. *Arch. Path.* **65,** 18.

FARR, R.S. (1958) A quantitative immunochemical measure of primary interaction between I BSA and antibody. *J. infect. Dis.* **103,** 239.

FELTKAMP-VROOM, T.M. & BOODE, J.H.M. (1970) An embedding and sectioning technique for

immunohistochemical studies of minute specimens of tissue. *J. clin. Path.* **23,** 188.

FELTKAMP-VROOM, T.M., STALLMAN, P., AALBERSE, R. & REERINK-BRONGERS, E.E. (1975) Immunofluorescence studies on renal tissue, tonsils, adenoids, nasal polyps and skin of atopic and non-atopic patients with special reference to IgE. *Clin. Immunol. Immunopath.* (In press.)

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

KOFFLER, D. AGNELLO, V. & KUNKEL, H.G. (1974) Polynucleotide immune complexes in serum and glomeruli of patients with systemic lupus erythematosus. *Amer. J. Path.* **74,** 109.

KOFFLER, D., SCHUR, P.H. & KUNKEL, H.G. (1967) Immunological studies concerning the nephritis of systemic lupus erythematosus. *J. exp. Med.* **126,** 607.

MANNIK, M. & AREND, W.P. (1971) Fate of preformed immune complexes in rabbits and rhesus monkeys. *J. exp. Med.* **134,** 19s.

MOLENAAR, J.L., MÜLLER, M.A.C., ENGELFRIET, C.P. & PONDMAN, K.W. (1974) Changes in antigenic properties of human C3 upon activation and conversion by trypsin. *J. Immunol.* **112,** 1444.

OROZCO, J.H., JASIN, H.E. & ZIFF, M. (1970) Defective phagocytosis in patients with systemic lupus erythematosus. *Arthr. Rheum.* **13,** 342.

PARKER, R.L. & SCHMID, F.R. (1962) Phagocytosis of particulate complexes of gammaglobulin and rheumatoid factor. *J. Immunol.* **88,** 519.

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

RAWSON, A.J., ABELSON, A.M. & HOLLANDER, J.L. (1965) Studies on the pathogenesis of rheumatoid joint inflammation. II. Intracytoplasmic particulate complexes in rheumatoid synovial fluids. *Ann. intern. Med.* **62,** 281.

ROBITAILLE, P. & TAN, E.M. (1973) Relationship between deoxyribonucleoprotein and deoxyribonucleic acid antibodies in systemic lupus erythematosus. *J. clin. Invest.* **52,** 316.

ROOS, C.M., FELTKAMP-VROOM, THEA M. & HELDER, A.W. (1975) The localization of hepatitis B antigen and immunoglobulin G in liver tissue. An immunofluorescence, light and electron microscopic study. *J. Path.* (In press.)

ROOS, D. & LOOS, J.A. (1970) Changes in the carbohydrate metabolism of mitogenically stimulated human peripheral lymphocytes. *Biochim. biophys. Acta (Amst.),* **222,** 565.

SMIT, J.W., MEIJER, C.J.L.M., DÉCARY, F. & FELTKAMP-VROOM, T.M. (1974) Paraformaldehyde fixation in immunofluorescence and immunoelectron microscopy. *J. immunol. Meth.* **6,** 93.

THE, T.H. & FELTKAMP, T.E.W. (1970) Conjugation of fluorescein isothiocyanate to antibodies. II. A reproducible method. *Immunology,* **18,** 875.

UDENFRIEND, S. (1969) *Fluorescence Assay in Biology and Medicine,* volume 2, p. 388. Academic Press, New York and London.

VAUGHAN, J.H., BARNETT, E.V., SIBEL, M.V. & JACOX, R.F. (1968) Intracytoplasmic inclusions of immunoglobulins in rheumatoid arthritis and other diseases. *Arthr. Rheum.* **11,** 125.

CHAPTER III

Immune complex detection by immunofluorescence on polymorphonuclear leucocytes.

Immune complex detection by IFT on PMN.

J.W.Steffelaar, F.J.W. ten Kate, M.Nap, A.J.G. Swaak, Claire B. de Graaff-Reitsma, E.H.van Elven and Thea M. Feltkamp-Vroom.

Department of Clinical Pathology, Erasmus University, Rotterdam.

and

Department of Immunohistopathology and Department for Autoimmune Diseases of the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service and Laboratory of Experimental and Clinical Immunology, University of Amsterdam.

Accepted for publication in Clinical and Experimental Immunology.

In this chapter a study of the influence of several anticoagulants, used for PMN isolation, on the occurrence of cytoplasmic Ig and C inclusions in these cells is reported. In addition, the influence of the antigen-antibody ratio on the in vitro phagocytosis of immune complexes was studied. For this purpose, immune complexes prepared from human HBsAg containing serum and human anti HBs containing serum were used.

SUMMARY

Polymorphonuclear leucocytes (PMN) from patients with systemic lupus erythematosus (SLE) were isolated from defibrinated and heparinized blood. In addition, PMN from a healthy donor were incubated with sera from SLE patients and with sera containing artificially prepared immune complexes of hepatitis B surface antigen (HBsAg) and human anti-HBsAg immunoglobulin (anti-HBs) with well defined variations of the antigen/antibody ratio. To one group of blood samples, 5 mM monoiodine acetic acid (MIAA) was added to block in vitro phagocytosis.

The PMN were examined for the presence of IgG, IgM, and HBsAg by the immunofluorescence technique. PMN from defibrinated blood of SLE patients showed in up to 80% immunoglobulin (Ig)-inclusions. However, addition of 5 mM MIAA reduced the number of Ig-containing PMN to at most 40%, which levels were equal to numbers found in specimens from heparinized blood. Addition of 5 mM MIAA to heparinized blood did not reduce the number of PMN with Ig inclusions. Normal donor PMN isolated from defibrinated heparinized, and EDTA blood showed equal amounts of Ig inclusions after incubation with SLE sera, but none when MIAA had been added. In PMN incubated with HBsAg-anti HBs immune complexes with an antigen antibody ratio between 5 and 0.2, both HBsAg and IgG could be detected.

It is concluded that Ig inclusions in PMN from heparinized blood from SLE patients are due to in vivo phagocytosis, presumably of circulating immune complexes. In vitro phagocytosis of Ig from SLE sera by normal donor PMN also suggests the presence of immune complexes.

Dependent on the antigen-antibody ratio, artificial HBsAg/anti HBs immune complexes can be detected by in vitro phagocytosis by PMN.

INTRODUCTION

Immunofluorescence studies in polymorphonuclear leucocytes (PMN) isolated from venous blood of patients with systemic lupus erythematosus (SLE), have demonstrated cytoplasmic inclusions composed of immunoglobulins (Ig) and complement components (C) presumably indicating immune complex phagocytosis (Vaughan et al. 1968; Cats, Lafeber and Klein 1975; Steffelaar, de Graaff-Reitsma and Feltkamp-Vroom 1976). However, PMN isolated from heparinized blood from SLE patients showed fewer and smaller inclusions than PMN isolated from defibrinated blood of the same patients (Steffelaar et al. 1976). Therefore, some of the inclusions in the PMN from defibrinated blood might represent in vitro phagocytosis of artificial immune complexes, possibly originating from cellular destruction by defibrination in the presence of anti nuclear antibodies (ANA).

The finding of in vitro phagocytosis by normal donor PMN of Ig and C from the sera of patients with rheumatoid arthritis and SLE (Cats et al. 1975) suggests that not all inclusions are caused by artificial immune complexes. The use of the in vitro technique and incubation of normal donor PMN with the sera to be tested for the presence of immune complexes, facilitates the study of factors with an influence in immune complex phagocytosis by PMN. The purpose of the present study was to find out whether immune complex phagocytosis occurs: 1. in vivo by peripheral blood PMN, studied in SLE patients (direct test), and 2. in vitro by normal donor PMN incubated with SLE sera (indirect test) as detected by the immunofluorescence technique. In addition, the influence of the antigen/

antibody ratio on the in vitro phagocytosis of artificially prepared immune complexes was studied.

Because of the possible pathogenetic role of immune complexes in glomerulonephritis, arthritis and vasculitis, described in association with human hepatitis B (Combes et al. 1971; Myers et al. 1972; Wands et al. 1975; Sergent et al. 1976), immune complexes, prepared from human serum containing hepatitis B surface antigen (HBsAg) and human anti-HBsAg immunoglobulin (anti-HBs) containing serum were used for this purpose.

MATERIALS AND METHODS

Subjects

The study was done in five patients with SLE diagnosed according to the criteria proposed by the American Rheumatism Association (Cohen et al. 1971). All five were in an active stage of the disease when the blood was sampled. PMN were isolated from this blood as described below (direct test), and the sera were used for the study of in vitro phagocytosis by normal donor PMN (indirect test). PMN for the in vitro studies were isolated from a healthy donor (bloodgroup O) with negative serological tests for HBsAg and anti-HBs.

Polymorphonuclear leucocytes

PMN were isolated with a slight modification of the technique described previously (Steffelaar et al. 1976): 10 ml of blood was either defibrinated, by shaking gently with glass beads for 10 min, heparinized (Thromboliquine, Organon, Oss, the Netherlands) or decalcified by addition of 6 mM EDTA. Blood samples were also heparinized and subsequently shaken with glass beads like the defibrinated

samples. One half of each blood sample was collected in 5 mM MIAA (pH 7.2) in order to block in vitro phagocytosis. After sedimentation with dextran (molecular weight 200,000) (Poviet, Amsterdam, the Netherlands) at 37oC for 30 min, the supernatant was layered on top of 1.5 ml Lymphoprep (Nyegaard, Oslo, Norway). After centrifugation at 20oC for 40 min at 400 g, the supernatant was discarded and the pellet was washed once by resuspension in washing fluid as described by Hijmans, Schuit and Klein (1969) and centrifuged for 10 min at 90 g. The recovery of PMN from heparinized blood was at least 95%. From the samples shaken with glass beads, about 20 per cent fewer PMN were harvested.

The PMN from the SLE patients were fixed, washed twice and frozen in a gelatin capsule in liquid nitrogen as described elsewhere (Steffelaar et al. 1976).

Phagocytosis in vitro.

a. After PMN isolation from donor blood (indirect test A)

After Lymphoprep-centrifugation, the pellets (each obtained from 5 ml of donor blood) were washed by resuspension in 5 ml of washing fluid and centrifugation for 10 min at 90 g. The pellets were then resuspended in 12 drops of washing fluid and 10 drops of the sera to be tested were added as well as 2 drops of the donor serum. After incubation at 37oC for 20 min, the suspensions were centrifuged for 10 min at 90 g and the pellets were washed, fixed, and frozen as described for the patient PMN.

b. Before PMN isolation from donor blood (indirect test B)

Three ml of donor blood were collected in 1.5 ml of SLE-serum to which heparin, glass beads or both had been added. The samples containing glass beads were gently

shaken for 10 min. From these blood-serum samples PMN were isolated, fixed and frozen as described above.

ANA-Titres

The ANA-titres were determined in the SLE-sera by the immunofluorescence technique. Cryostat sections of normal rat liver tissue were incubated with several dilutions of the sera, followed by washing in PBS (pH 7.2) and subsequent incubation with FITC-labeled horse-anti-human immunoglobulin (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, the Netherlands). After isolation of PMN, the ANA-titres, corrected for a dilution factor caused by the PMN isolation procedure, were also determined in the remaining serum and plasma samples of the SLE-patients.

Preparation of artificial immune complexes.

HBsAg containing serum was obtained from a patient with hepatitis B. The HBsAg titre was 1:6,400, determined with the haemagglutination inhibition technique (Reesink, Duimel and Brummelhuis, 1973). Anti-HBs containing serum was collected from a reconvalescent hepatitis B patient. In the haemagglutination technique (Reesink et al. 1973) the anti-HBs titre was 1:51,200.

The equivalence ratio of this HBsAg/anti-HBs serum pair was determined by mixing them in different proportions and applying the agar gel diffusion test with HBsAg and anti-HBs. The equivalence ratio was defined as the HBsAg/anti-HBs ratio for which the sample reacted with neither HBsAg nor anti-HBs. The equivalence ratio was said to be 1, and was used as the reference point to obtain immune complexes with HBsAg/anti-HBs ratios ranging from 10 to 0.01. The antigen excess immune complexes were prepared by adding HBsAg containing serum to a constant

amount of anti HBs containing serum. The antibody excess immune complexes were prepared by adding anti HBs to a constant amount of HBsAg containing serum. The sera were rapidly mixed, followed by an incubation period of two hours at room temperature.

Immunofluorescence procedure

The indirect immunofluorescence technique was used for the demonstration of IgG and IgM and the number of cells with fluorescent inclusions were counted as previously described (Steffelaar et al. 1976). The presence of HBsAg was determined with the direct technique, using rabbit-anti-HBsAg antiserum (Roos, Feltkamp-Vroom and Helder 1976) conjugated to fluorescein isothiocyanate (FITC) (The and Feltkamp 1970) and adsorbed with sonificated donor PMN.

RESULTS

Direct and indirect tests in SLE patients

Immunofluorescence studies revealed two types of PMN-inclusions containing IgG or IgM: small granular inclusions and occasional large (7-12 mu) homogeneous inclusion bodies as described elsewhere (Steffelaar et al. 1976). The large inclusions were only present in PMN from either defibrinated blood samples or heparinized samples to which glass beads had been added, both in the direct test and in de indirect test B, but not in de indirect test A. However, in the samples containing MIAA, no large inclusions could be detected.

Table 1 shows the percentages of PMN with small granular inclusions containing IgG or IgM for the SLE patients (direct test and indirect test A).

In the indirect test B the percentage of PMN with

Table I

Percentage of PMN showing small granular Ig-inclusions in the direct test and indirect test A for five SLE patients

Patient		1				2				3				4				5			
Inclusions		IgG		IgM		IgG		IgM		IgG		IgM		IgG		IgM		IgG		IgM	
5 mM MIAA →		D	I	D	I	D	I	D	I	D	I	D	I	D	I	D	I	D	I	D	I
Defibrination	-	40	5	30	10	80	20	70	20	50	10	40	10	5	0	40	15	80	10	70	20
	+	5	0	10	0	40	0	30	0	10	0	15	0	0	0	20	0	20	0	20	0
Heparin	-	5	5	10	10	40	20	30	20	10	10	15	10	0	0	20	15	20	10	20	20
	+	5	0	10	0	40	0	30	0	10	0	15	0	0	0	20	0	20	0	20	0
EDTA	-	-	5	-	10	-	20	-	20	-	10	-	10	-	0	-	15	-	10	-	20
	+	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0

D , I = direct test or indirect test A.

granular Ig-containing inclusions were the same as in the indirect test A, when the PMN were isolated from heparinized blood. However, when isolated from defibrinated blood, or when glass beads were added to heparinized samples with subsequent shaking, the percentages rose to 60-80% in all cases. The donor PMN contained no inclusions in the direct test, or in the indirect test when incubated with either SLE serum in the presence of MIAA or with normal human serum.

ANA-Titres

All SLE-patients showed ANA-titres varying from 32 to 128. No significant reduction in the ANA-titres was demonstrated after defibrination and subsequent PMN-isolation. However, the samples containing heparin showed a marked reduction of the ANA-titres.

Phagocytosis of artificially prepared immune complexes

PMN incubated with HBsAg/anti HBs immune complexes with an antigen/antibody ratio ranging from 5 to 0.2 showed inclusions composed of both HBsAg and Ig, as Table 2 shows. The number and size of the inclusions were arbitrarily scored as +, ++ or +++. However, neither HBsAg nor Ig was detected in the PMN when the antigen/antibody ratio of the immune complexes was 8 or more, and only Ig was demonstrated when this ratio ranged from 0.1 to 0.01.

DISCUSSION

The results of immunofluorescence studies on PMN isolated from the blood of SLE patients (direct test) are dependent on the isolation procedure used. When isolated from defibrinated blood, up to 80% of the PMN show small granular inclusions composed of immunoglobulins of several

Table 2

In vitro phagocytosis of HBsAg-anti HBs immune complexes and variation in the Ag/Ab ratio.

Ratio HBsAg / anti-HBs	Immunofluorescence RaHBsAg^{Fl} o)	a IgG +) a IgM
10	-	-
8	-	-
5	+	+
3	+	+
2	++	++
1	++	++
0.5	+++	+++
0.33	++	+++
0.2	+	+++
0.1	-	++
0.05	-	++
0.02	-	+
0.01	-	+
Controls		
HBsAg	-	-
anti HBs	-	-
NHS*	-	-

* NHS = normal human serum

o) = rabbit anti HBsAg antiserum conjugated to FITC

+) = rabbit anti human IgG and rabbit anti human IgM antisera

conditions of slight antibody excess.

To our knowledge there are no published reports concerning the pathogenic properties of HBsAg/anti HBs immune complexes in relation to their antigen/antibody ratio. However, the relative quantities of antigen and antibody have been demonstrated under many experimental conditions to be of crucial importance for the pathogenecity of the resulting immune complexes. In both the one shot serum sickness model and experimental chronic serum sickness slight antigen excess results in the formation of pathogenic immune complexes causing glomerular and vascular lesions (Dixon et al. 1958, 1971). Viral infections may correspond with this experimental situation, and persistent viremia, which induces a low-level continuous immune response resulting in glomerulonephritis, has been reported in the course of chronic viral infections in various animals (Oldstone and Dixon 1971).

Glomerulonephritis (Combes et al. 1971; Myers et al. 1972; Knieser et al. 1974; Kohler et al. 1974), arthritis (Wands et al. 1975), and vasculitis (Sergent et al. 1976) have also been described in human hepatitis B patients, and are presumably due to immune complex deposition.

It may be postulated that in human hepatitis B too, approximate balance of the HBsAg and anti HBs production results in pathogenic immune complexes. Several authors have reported the demonstration of HBsAg-anti HBs immune complexes in serum from hepatitis B patients (Shulman and Barker, 1969; Nydegger et al. 1974; Wands et al. 1975; Theofilopoulos, Wilson and Dixon, 1976), but others failed to detect such complexes (Sergent et al. 1976).

The results reported here indicate that complexes formed under conditions of approximate balance of HBsAg and anti HBs are readily demonstrated on the basis of in vitro phagocytosis by PMN. Since these complexes are likely to be pathogenic when occurring in vivo, it would be worthwhile to find out whether HBsAg and Ig inclusions

can be demonstrated in peripheral-blood PMN of hepatitis B patients with signs and symptoms of arthritis, vasculitis, or glomerulonephritis.

REFERENCES

Cats, A., Lafeber, G.J.M. and Klein, F. (1975). Immunoglobulin phagocytosis by granulocytes from sera and synovial fluids in various rheumatoid and non rheumatoid diseases. Ann. Rheum. Dis. 34, 146.

Cohen, A.S., Reynolds, W.E., Franklin, E.C., Kulka, J.P., Ropes, M.W., Schulman, L.E. and Wallace, S.L. (1971). Preliminary criteria for the classification of systemic lupus erythematosus. Bull. rheum. Dis. 21, 643.

Combes, B., Stastny, P., Shorey, J., Eigenbrodt, E.H., Barrera, A., Hull, A.R., and Carter, N.W. (1971). Glomerulonephritis with deposition of Australia antigen-antibody complexes in glomerular basement membrane. Lancet 2: 234.

Dixon, F.J., Vazquez, J.J., Weigle, W.O., and Cochrane, C.G., (1958). The pathogenesis of serum sickness. Arch. Path. 65, 18.

Dixon, F.J. (1971). Mechanisms of immunologic injury. In: Immunobiology. Editors: Good, R.A. and Fisher, D.W., Sinauer Associates, Inc., Stamford, Connecticut.

Hijmans, W., Schuit, H.E. and Klein, F., (1969). An immunofluorescence procedure for the detection of intracellular immunoglobulins. Clin. Exp. Immunol. 4, 457.

Knieser, M.R., Jenis, E.H., Lowenthal, D.T., Bancroft, W.H., Burns, W., and Shalhoub, R. (1974).Pathogenesis of renal disease associated with viral hepatitis. Arch. Pathol. 97, 193.

Kohler, P.F., Cronin, R.E., Hammond, W.S., Olin, D. and Carr, R.I. (1974). Chronic membranous glomerulonephritis caused by hepatitis B antigen-antibody immune complexes. Ann. Intern. Med. 81, 448.

Myers, B.D., Griffel, B., Naveh, D., Jankielowitz, T. and Klajman, A. (1973). Membranoproliferative glomerulonephritis associated with persistent viral hepatitis. Am. J. Clin. Path. 60, 222.

Nydegger, U.E., Lambert, P.H., Gerber, H., and Miescher, P.A. (1974). Circulating immune complexes in the serum in systemic lupus erythematosus and in carriers of hepatitis B antigen. Quatitation by binding to radiolabeled Clq. J. Clin. Invest. 54, 297.

Oldstone, M.B.A. and Dixon, F.J. (1971). Immune complex disease in chronic viral infections.J.Exp.Med.134,325.

Reesink, H.W., Duimel, W.J.M. and Brummelhuis, H.G.J.(1973) Standardization and modification of the haemagglutination (inhibition) techniques for the determination of HBAg and anti-HBAg antibodies. Vox Sang. 24, 528.

Roos, C.M. Feltkamp-Vroom, Th.M., and Helder, A.W. (1976). The localization of hepatitis B antigen and immunoglobulin G in liver tissue; an immunofluorescence, light and electronmicroscopic study. J. Path. 118, 1.

Sergent, J.S., Lockshin, M.D., Christian, C.L. and Gocke, D.J. (1976). Vasculitis with hepatitis B antigenemia. Long term observations in nine patients. Medicine, 55, 1.

Shulman, N.R. and Barker, L.F. (1969). Virus-like antigen, antibody and antigen-antibody complexes in hepatitis measured by complement fixation. Science, 165, 304.

Steffelaar, J.W., de Graaff-Reitsma, C.B. and Feltkamp-Vroom, Th.M. (1976). Immune complex detection by immunofluorescence on peripheral blood polymorphonuclear leucocytes. Clin. Exp. Immunol. 23, 272.

The, T.H. and Feltkamp, T.E.W. (1970). Conjugation of fluorescein isothiocyanate to antibodies. II A reproducible method. Immunology 18, 875.

Theofilopoulos, A.N., Wilson, C.B. and Dixon, F.J. (1976). The Raji cell radioimmune assay for detecting immune complexes in human sera. J. Clin. Invest. 57, 169.

Vaughan, J.H., Barnett, E.V., Sobel, M.V. and Jacox, R.F. (1968). Intracytoplasmic inclusions of immunoglobulins in rheumatoid arthritis and other diseases. Arthr. Rheum. 11, 125.

Wands, J.R., Mann, E.,,Alpert, E. and Isselbacher, R.J. (1975). The Pathogenesis of arthritis associated with acute hepatitis-B surface antigen positive hepatitis. Complement activation and characterization of circulating immune complexes. J. Clin. Invest. 55, 930.

Zebrowski, A. (1972). Studies on the effect of heparin on detectability of antinuclear antibodies by immunofluorescent method in sera of visceral lupus patients. II. The effect of heparin on antibody titre. Ann. Immunol. (Poznan) 4, 79.

CHAPTER IV

HBsAg AND SYSTEMIC DISEASE. REVIEW OF THE LITERATURE.

INTRODUCTION

Several studies (Kohler, 1973; Gocke, 1975) have drawn attention to extrahepatic manifestations of viral hepatitis and the role of circulating immune complexes in the pathogenesis of these syndromes. Gocke (1975) recognized three types of syndromes: first a serum sickness like prodrome of acute hepatitis consisting of urticaria, rash, polyarthralgia and sometimes arthritis. Secondly he noted an incidence of 30-40% of HBsAg antigenaemia in patients with PAN, and thirdly, an immune complex type of glomerulonephritis, usually in association with chronic active hepatitis.

In this chapter, the literature concerning systemic manifestations of hepatitis B is reviewed, as well as the techniques used for detection of circulating HBsAg-anti HBs immune complexes.

HBsAg AND VASCULITIS

A high incidence of HBsAg has been demonstrated in the serum of patients with PAN (Prince and Trepo, 1971; Gocke et al., 1971; Trepo, Zuckerman and Prince, 1974). However, the role of HBsAg-anti HBs immune complexes in the pathogenesis of vasculitis is still controversial.

Gocke et al. (1971) demonstrated both HBsAg and Ig in vessel walls by the IFT in two out of six patients with combined PAN and HBsAg-positive serology. In these two patients and in one patient without HBsAg or Ig in affected vessel walls, evidence of circulating immune

complexes was obtained by lowered CH50-values and by the C1q-precipitation reaction as described by Agnello, Winchester and Kunkel (1970). In the same study, in the five patients tested, 20 and 40 nm particles were demonstrated by electron microscopic examination of serum pellets after ultracentrifugation. These particles were interpreted as characteristically associated with HBsAg. However, the pellets were negative by immunologic techniques for HBsAg, and aggregation of the particles, indicative of immune complex formation (Almeida and Waterson, 1969) was not reported.

Prince and Trepo (1971) reported 4 HBsAg seropositive cases out of 15 patients with PAN. Three more patients had detectable anti HBs. No IF-studies were reported on the biopsy specimens. In one out of four serum specimens from the HBsAg seropositive patients with PAN immune complexes were demonstrated by density gradient centrifugation. The authors concluded that the existence of these complexes might be an epiphenomenon unrelated to the primary pathogenetic mechanism.

Heathcote, Dudley and Sherlock (1972) reported five HBsAg seropositive patients with chronic hepatitis and PAN. In this study no complexes of HBsAg and antibody in the sera were seen with the electron microscope and no such complexes were seen in the affected vessel walls by IF. However, low serum levels of C4, anticomplementary activity of the sera from two patients and Ig- and C- deposits in vessel walls in two patients suggested that immune complexes apparently not containing HBsAg were involved in the pathogenesis of the vasculitis in these patients.

Gerber et al. (1972) reported a patient with lymphatic leucaemia, who developed active PAN one month after recovery of an acute hepatitis B. No circulating immune complexes were detected in the serum neither with the use of the C1q precipitation technique (Agnello et al. 1970), nor by electron microscopic examination of serum

pellets after ultracentrifugation. IF-studies of arteries with PAN demonstrated HBsAg in the lesions, without the presence of Ig or C. Since HBsAg was also present in renal infarctions, the authors suggested that these deposits might be secondary to tissue damage.

Baker et al. (1972) reported one patient with PAN subsequent to HBsAg positive hepatitis. IF-studies showed HBsAg in the walls of the affected vessels; however, Ig or C were not demonstrated. Serum complement levels were normal. Electron microscopic examination of serum pellets showed 20 nm particles as described in association with HBsAg. Clumping of these particles was not demonstrated.

Kohler (1973) reported the presence of HBsAg in the serum of one patient with PAN and anti-HBs in another in a series of 7 consecutive cases of PAN. In IF-studies of autopsy tissue of two cases, no HBsAg could be demonstrated in the vessel walls.

Trepo et al. (1974) reported serological studies on 55 cases of PAN. Histological data and IF-studies of the affected vessels were not reported. HBsAg was detected in 30 patients and anti-HBs in 13 patients. In 5 out of these cases both HBsAg and anti-HBs were present. In one of four cases from this series lowered complement levels and raised anticomplementary activity was reported. Electron microscopic detection of clumps of HBsAg-associated particles in serum pellets were considered to represent circulating immune complexes, and were demonstrated in 12 out of 18 cases with active vasculitis. No clumping was demonstrated in two cases with quiescent vasculitis. The authors concluded that circulating HBsAg-anti HBs complexes might be responsible for PAN. However, no correlation was found between the presence of immune complexes and the type of the hepatitis.

Sergent et al. (1976) reported HBsAg-positive serology in nine out of 21 patients with PAN. Four cases were presented in an earlier report (Gocke et al., 1970).

Except for one renal biopsy specimen without signs of PAN, no IF-studies on biopsy specimens were reported. In 3 cases serum complement levels were determined during active vasculitis and found to be less than 30% in one. In sera from 5 cases obtained during active periods of vasculitis immune complexes could not be demonstrated by the Clq precipitation reaction (Agnello et al., 1970) nor by double diffusion in gel against purified IgM rheumatoid factor (Winchester et al., 1971) Also ultracentrifugation studies using ^{125}I-HBsAg added to patients sera failed to demonstrate immune complexes, and none of the patients showed cryoglobulinaemia.

Histologic data concerning liver disease in hepatitis B patients with associated PAN, summarized in table 1, were presented by Trepo, Thivolet and Lambert (1972) (2 cases), Heathcote et al. (5 cases), Nowoslawski et al. (6 cases), Gerber et al. (1 case), Baker et al. (1 case), Sergent et al. (9 cases) and Duffy et al. (10 cases). These data indicate that no significant relation of PAN to any of the histological types of hepatitis can be recognized.

HBsAg AND ARTHRITIS

The incidence of joint manifestations in viral hepatitis, as given by several authors varies from 1.6% to 49%, according to the manner in which it was sought (reviewed by Alarcon and Townes, 1973). In 20.6% of patients with hepatitis a history of arthralgia could be elicited after specifically asking for it (Mosley and Galambos, 1969). However, Alarcon and Townes (1973) collected only 57 cases with objective signs of arthritis (i.e. redness, swelling or increased local temperature) from an extensive study of the literature, including their own 5 cases. In all cases it was characterized as a self-

Table 1

Histological diagnoses on liver biopsy specimens from hepatitis B patients with associated PAN, compiled from the literature (see text).

Diagnosis	No. of cases
AcH	6
CPH	8
CAH	7
Cirrhosis	2
Aspecific	5
"Mild hepatitis"	2
"Moderate hepatitis"	1
"Subacute hepatitis"	1
"Fulminant hepatitis"	1
Septal fibrosis	1
	Total 34

limiting disease of about three weeks duration.

Kohler (1973) reported an overall incidence of 29% of immune complex symptoms in 100 cases of acute hepatitis B and noted that these symptoms occurred significantly more frequently in female compared to male patients (48% and 15% respectively). The symptoms included arthralgia-arthritis and urticaria-rash, and occurred predominantly during the prodromal phase of hepatitis. No vasculitis or glomerulonephritis was reported in this series.

Support for the hypothesis that circulating immune complexes are a major pathogenetic factor in serum hepatitis-associated arthritis was first derived from complement component studies (Alpert, Isselbacher and Schur, 1971; Onion, Crumpacker and Gilliland, 1971). Alpert et al. (1971) reported low serum levels of CH50, C4 and C3 in eight out of nine patients with active arthritis, in association with high titres of HBsAg.In contrast to these findings in patients with acute joint symptoms, normal or elevated complement component levels were demonstrated in patients during convalescence from these symptoms or without signs of arthritis. No anti-HBs was detected in any of the patients with and without arthritis. Sequential studies of serum complement levels in the course of HBsAg-related arthritis have been reported and were interpreted as additional evidence of the role of circulating immune complexes in this disease (Onion et al., 1971; Wenzel et al., 1972; Alpert, Schur and Isselbacher, 1972). However, normal serum complement levels during the acute phase of hepatitis-associated arthritis have also been reported (Alpert et al., 1971; McCarty and Ormiste, 1973; Duffy et al., 1976) in 20 out of 42 cases.

Wands et al.(1975) analyzed the occurrence and composition of cryoprecipitable proteins in 6 patients with acute HBsAg-positive hepatitis, in three cases complicated by arthritis. In most sera the antigen titer was raised several fold in the cryoprecipitate as compared

to whole serum titres. In one case complicated by arthritis the simultaneous occurrence of HBsAg and anti HBs was demonstrated in low titres in the cryoprecipitate, whereas in whole serum a high titre of antigen was demonstrated without detectable antibody. Both HBsAg and anti HBs disappeared from cryoprecipitate and whole serum as the arthritic symptoms subsided. Sequential studies revealed the successive occurrence of antigen and antibody in the expected order in two cases; in two cases only antigen was demonstrated and in one case only antibody in the period studied.

HBsAg AND GLOMERULONEPHRITIS

The incidence of glomerulonephritis in association with chronic active hepatitis of unknown etiology has been reported to be 7% (Mistilis and Blackburn, 1970). Eknoyan et al. (1971) studied renal biopsy specimens from 7 patients with acute viral hepatitis. Four out of these patients were HBsAg positive. Lightmicroscopical changes were described as of very minor degree. IF-study demonstrated focal nodular deposits of IgG, IgM, IgA and B1C in all seven biopsy specimens. However, the presence of HBsAg was not tested by the IF-technique. Electron microscopic examination revealed tubular structures as described in association with systemic lupus erythematosus, but no HBsAg-associated particles were demonstrated.

Randall et al. (1971) reported six cases of renal manifestations in patients with chronic hepatitis. Antibodies against HBsAg were not present in any of the patients; the presence of HBsAg was not reported. All cases showed membranoproliferative glomerulonephritis. Out of four biopsy specimens available for IF-study, two showed linear fluorescence of the glomerular basement membrane (GBM), when tested with anti-immunoglobulin antisera, and all specimens showed lumpy glomerular

deposits of complement. Serum antibodies were present against human GBM in two cases, against nuclear protein in five and against smooth muscle in two. Serum complement levels were lowered in five cases.

The first well-documented case of hepatitis-B associated glomerulonephritis with evidence of a causal relationship of HBsAg and renal disease was reported by Combes et al. (1971). IF-studies revealed granular deposits of IgG, Complement and HBsAg along the glomerular basement membrane in a renal biopsy specimen showing membranous glomerulonephritis. However, circulating immune complexes could not de demonstrated in this case, using sucrose-gradient ultracentrifugation and C1q-precipitation techniques. In addition serum complement levels were normal, cryoglobulinaemia was not detected and serum anticomplementary activity was not present.

Nowoslawski et al. (1972) studied tissue specimens obtained at autopsy from 29 patients who died in the course of fulminant hepatitis, subacute hepatitis, chronic aggressive hepatitis, postnecrotic liver cirrhosis and chronic persistent hepatitis respectively. All cases were HBsAg positive. The incidence of clinical manifestations of renal disease in these patients was not reported. However, chronic membranous glomerulonephritis was diagnosed in four cases, and IF-studies revealed granular and lumpy deposits of HBsAg, IgG, IgM and B1C along the GBM in all four cases. In addition, HBsAg, IgG, IgM and B1C were demonstrated in small and large arteries showing lesions comparable with panarteritis in the healing stage.

Myers et al. (1973) reported a case of membranoproliferative glomerulonephritis associated with persistent HBsAg positive hepatitis. IF-studies revealed granular deposits of IgG, complement and HBsAg. Serum complement levels (C3 and C4) were low.

Three cases of immune complex glomerulopathy in association with HBsAg-positive hepatitis were reported

by Knieser et al. (1974). The glomerular changes were classified as membranous glomerulonephritis, focal glomerulosclerosis and membranoproliferative glomerulonephritis respectively. Evidence of deposition of HBsAg-containing immune complexes was derived from both IF-studies and immuno-electron microscopic examination of renal tissue biopsy specimens. Liver biopsy specimens from all cases showed chronic active hepatitis from the onset or in follow-up. Except for the case of membranoproliferative glomerulonephritis, which was reported to be normocomplementaemic, no serum complement levels were reported, nor studies concerning the detection of immune complexes in the serum.

Kohler et al. (1974) reported one case of membranous glomerulonephritis with deposition of immune complexes containing HBsAg, as demonstrated by the IF-technique. Electron microscopic study did not reveal any particles morphologically resembling the spherical 20 nm form of HBsAg. A liver biopsy specimen showed chronic active hepatitis. Serological tests related to the detection of circulating immune complexes included study of complement levels of the components C1q, C4, C3 and C5, rheumatoid factor, antinuclear antibodies, C1q precipitation and cryoproteins. Serial samples taken during 12 months showed normal complement levels. C1q precipitins were not detected and tests for rheumatoid factor and antinuclear antibodies were consistently negative. The analysis of cryoproteins demonstrated anti-HBs immunoglobulins at a 1:32 titre, whereas whole serum was negative for antibody. HBsAg was detected in a cryoprecipitate concentration of less than 5% of that in whole serum. In addition, IgG, IgM and C3 were demonstrated in the cryoprecipitate. These findings were interpreted as evidence of circulating HBsAg-anti HBs immune complexes in this patient. A second patient with HBsAg-anti HBs immune complex glomerulonephritis was identified by these authors, who reported

a prevalence of 1.4% (2 out of 145 renal biopsies).

Brzosko et al. (1974) reported immune complex glomerulonephritis in 32 out of 52 unselected kidney biopsy specimens from children. In 18 out of these 32 cases with immune complex glomerulonephritis HBsAg was identified in the glomerular lesions, in addition to Ig and C. In most cases, the glomerular lesions were classified as membrano-proliferative glomerulonephritis (12/18). In addition, two cases of membranous, intracapillary and extracapillary proliferative glomerulonephritis respectively were reported.

McIntosh et al. (1976) reported 3 cases of membranous glomerulonephritis with glomerular deposition of HBsAg and Ig. No histological data concerning the associated type of hepatitis were reported.

A summary of the histological diagnoses on renal and liver biopsy specimens in cases of hepatitis B associated glomerulonephritis with glomerular HBsAg containing deposits, demonstrated by IF, is given in table 2. These data indicate that glomerulonephritis with HBsAg-deposition in the glomeruli occurs predominantly in association with CAH.

DETECTION TECHNIQUES USED FOR HBsAg-ANTI HBs IMMUNE COMPLEXES

Shulman and Barker (1969) demonstrated anticomplementary (AC) activity of the serum at some time during hepatitis in 95% of 130 volunteers exposed parenterally to blood products known to transmit viral hepatitis. This AC-activity was attributed to immune complexes consisting of HBsAg and antibody. Further evidence that antigen-antibody complexes accounted for the AC-activity was provided by the ability of excess antigen or antibody

Table 2

Histological diagnoses on renal and liver biopsy specimens in cases of hepatitis B associated glomerulonephritis with glomerular HBsAg containing depositions demonstrated by IF.

Author	Renal biopsy specimen	No of cases	Liver biopsy specimen	No of cases
Combes et al. (1971)	membranous GN	1	AcH (→CPH?)	1
Nowoslawski et al. (1972)	membranous GN	4	CAH	3
			"fulminant hepatitis"	1
Myers et al. (1973)	membranoproliferative GN	1	CAH (+ cirrh.)	1
Knieser et al. (1974)	membranoproliferative GN	1		
	membranous GN	1	CAH	3
	focal glomerulosclerosis	1		
Kohler et al. (1974)	membranous GN	1	CAH	1
McIntosh et al. (1976)	membranous GN	3	not reported	
Brzosko et al. (1974)	membranoproliferative GN	12		
	membranous GN	2	not reported	
	endocapillary GN	2		
	extracapillary GN	2		

to reverse it. Although the activity of liver disease was mentioned, systemic manifestations attributable to circulating immune complexes were not reported.

Almeida and Waterson (1969) described in an electron microscopic study of the sera of HBsAg positive patients spherical and tubular particles with a diameter of 20-25 nm, the tubular structures having lengths that varied from 30 to 700 nm. In one subject without clinical signs of liver disease, these particles showed a random distribution. However, addition of rabbit-anti HBsAg immunoglobulin caused aggregate formation and clumping of these particles. In a patient with chronic active hepatitis both aggregated and at random distributed particles were demonstrated, representing HBsAg-anti HBs immune complexes in antigen excess. The serum from a fatal case of acute hepatitis contained numerous aggregates, interpreted as HBsAg-anti HBs immune complexes in antibody excess, since no free particles were seen in this specimen. Although the occurrence of immune complexes in the sera from these patients correlated well with the severity of liver disease, systemic disease due to circulating immune complexes was not reported.

Millman et al. (1970) mentioned the possibility that HBsAg may be undetectable by immunodiffusion in sera containing anti HBs. These authors reported the use of dissociation of immune complexes as a method for their detection. Immune complex dissociation was achieved by ultracentrifugation in cases of low affinity complexes and by enzyme treatment in cases of high affinity complexes.

The changes in physicochemical properties of HBsAg and anti-HBs after complex formation were used by Prince and Trepo (1971), who analyzed the sera from patients with hepatitis and polyarteritis nodosa by rate-zonal density gradient centrifugation. When antigen-containing sera were centrifuged through gradients containing

10-40% sucrose during 2 hours at 25,000 rpm the antigen sedimented as a single peak with a sedimentation coefficient of approximately 110 S. However, when sera containing both antigen and antibody were centrifuged through gradients of this type, antigen was found to sediment at varying rates between 110 S and 1000 S or more. The results of these studies are mentioned under "HBsAg and vasculitis".

The interaction of the C1q component of complement with aggregated immunoglobulin and immune complexes, resulting in C1q-precipitation, has been applied to the detection of immune complexes in sera from patients with SLE, rheumatoid arthritis and polyarteritis nodosa, using the agar gel diffusion method (Agnello, et al., 1970, Gocke et al., 1971).

McAuliffe, Kachani and Gocke (1973) reported a bioassay, based on the inflammatory reaction in the skin of guinea pigs after intradermal injections of serum fractions containing in vitro prepared HBsAg-anti HBs immune complexes. Both antigen-excess and equivalence-zone complexes were tested. Antigen-excess immune complexes produced an immediate increase in vascular permeability, whereas equivalence-zone precipitates showed no significant differences with controls.

Nydegger et al. (1974) described a method for immune complex detection based on both the C1q binding properties of immune complexes and on their large molecular size, using fractionated precipitation of immune complex bound and free ^{125}I C1q in polyethylene glycol (PEG). These authors studied 54 sera from 49 HBsAg-positive subjects. Eighteen healthy carriers did not exhibit increased ^{125}I C1q precipitation when compared to normal human donors. However, 4 out of 7 patients with chronic persistent hepatitis and 7 out of 24 patients with acute transient hepatitis had increased ^{125}I C1q precipitation values. It was stressed that no correlation between the increased ^{125}I C1q precipitation and the clinical status

of the patient could be demonstrated, and systemic disease due to circulating immune complexes was not reported.

Woolf et al. (1975) studied 36 sera from HBsAg-positive individuals, including cases of histologically confirmed cirrhosis, chronic aggressive and chronic persistent hepatitis and focal necrosis, as well as cases without histological damage of the liver. In all sera 20 nm particles predominated. However, aggregation of particles into complexes was not observed without the prior addition of anti-HBsAg antiserum.

Wands et al. (1975) analyzed the occurrence and composition of cryoprecipitable proteins in the sera from patients with HBsAg-positive hepatitis. The results of these studies are summarized under "HBsAg and arthritis".

Theofilopoulos, Wilson and Dixon (1976) reported the use of a human lymphoblastoid cell line with B cell characteristics, derived from a Burkitt lymphoma (Raji cells) as an in vitro detection technique for circulating immune complexes. These cells lack membrane bound immunoglobulin, but they have receptors for IgG Fc, C3b, C3d and C1q. Via the C-receptors immune complexes in human sera may be bound to the Raji cells and in turn quantitated by measuring the uptake of radiolabeled rabbit-anti-human antibody. The amount of immune complexes present is expressed as the amount of aggregated human immunoglobulin inducing an equivalent uptake of radiolabeled antibody. The antigens involved may be identified, using the immunofluorescence technique with FITC-labeled antisera, as was demonstrated with HBsAg-containing immune complexes The authors reported in 12 out of 19 patients with acute HBsAg-positive hepatitis the presence of circulating immune complexes, as well as in 8 out of 59 asymptomatic HBsAg carriers. The occurrence of systemic disease, related to circulating immune complexes was not reported.

McIntosh, Koss and Gocke (1976) studied the nature and incidence of cryoproteins in HBsAg positive patients.

Their results indicate that the amount of cryoprecipitable material is highest in acute hepatitis. Furthermore, these authors stressed that systemic disease (i.e. vasculitis and glomerulonephritis) occurred predominantly in patients with chronic hepatitis, the cryoprecipitate containing large amounts of antigen but little antibody. In contrast, in patients with chronic hepatitis without vasculitis, both antigen and antibody was detected in the cryoprecipitate.
In vitro studies, using artificially prepared immune complexes of HBsAg and human anti-HBs have demonstrated that HBsAg-anti HBs immune complexes can be detected by phagocytosis by PMN. The detectability of the immune complexes was demonstrated to be dependent on the antigen/antibody ratio, and both Ig and HBsAg were identified within the PMN, if this ratio ranged from 5 to 0.2 (Steffelaar et al., 1977).

From this review of the literature it is concluded that evidence of the presence of circulating immune complexes in hepatitis B patients has been presented by several authors, although involvement of HBsAg in immune complex formation was demonstrated in a minority of the studies reported. Several authors discussed the relation of liver damage and circulating immune complexes. However, the results of the immune complex detection techniques in relation to systemic manifestations, attributable to circulating immune complexes on one hand and histological characterization of the liver disease on the other hand, has not been analysed.

Chapter V

Hepatitis B surface antigen (HBsAg) and systemic disease. A clinico pathological study.

HBsAg and systemic disease.

J.W.Steffelaar, F.J.W. ten Kate, M. Nap, E.H. van Elven and M. van Blankenstein.

From the Department of Pathology and Internal Medicine, School of Medicine of the Erasmus University, Rotterdam, the Netherlands and the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, the Netherlands.

Submitted for publication.

In this chapter a study of systemic manifestations in relation to hepatitis B and the occurrence of circulating immune complexes is reported. The PMN phagocytosis test was used as parameter for the presence of immune complexes in the circulation.

SUMMARY

The occurrence of manifestations of systemic disease in 107 HBsAg seropositive subjects with histologically staged liver disease was screened. In addition, 93 cases of immune complex glomerulonephritis were screened for HBsAg-containing deposits in glomeruli and vessel walls. The presence of immune complexes in the serum of 35 HBsAg-seropositive subjects was tested by immunofluorescence study of normal donor polymorphonuclear leucocytes (PMN) after incubation with serum from these patients. In this series the incidence of arthralgia in the prodromal or early acute phase of hepatitis B was about 20%. Convincing evidence of the involvement of HBsAg in the pathogenesis of these joint symptoms was lacking. Polyarteritis nodosa (PAN) in association with chronic persistent hepatitis B was present in one case. Although the PMN phagocytosis test indicated the presence of circulating immune complexes, a causal relationship of the vasculitis and HBsAg could not be demonstrated. No case of HBsAg-associated glomerulonephritis was present in the material studied. The presence of circulating immune complexes was demonstrated by the PMN phagocytosis test only in cases of chronic active hepatitis, except for two cases of PAN, associated with chronic persistent hepatitis in one. Since 8 out of 10 cases of glomerulonephritis with glomerular HBsAg deposits have been reported in association with chronic active hepatitis, these data suggest that the PMN phagocytosis test is able to detect and discriminate potential pathogenic circulating immune complexes.

Arthritis, glomerulonephritis and vasculitis have been reported in the course of hepatitis associated with hepatitis B surface antigen (HBsAg) (2, 7, 11, 16, 24, 26, 28, 31, 34, 38, 46) Deposition of circulating immune complexes is supposed to be the cause of these complications, and the presence of both HBsAg and immunoglobulins (Ig) has been reported in biopsy specimens of the tissues involved (4, 7, 17, 24, 26, 29, 31, 32). However, as compared with a prevalence of 1.90 per 1,000 of HBsAg-associated hepatitis (42), well documented cases of systemic disease caused by deposition of HBsAg-containing immune complexes are rare, and very few cases have been published presenting evidence of HBsAg-containing immune complexes both in the serum and in renal or vascular tissues from the same patients.

Several authors have demonstrated the occurrence of HBsAg-containing immune complexes in the sera from up to 95% of the patients with HBsAg-associated hepatitis (29, 33, 35, 39, 44). Therefore only a small minority of the immune complexes detected by the techniques used can be relevant as far as immune complex deposition resulting in clinically manifest systemic disease is concerned.

In vitro studies, using artificially prepared immune complexes of HBsAg and human anti-HBsAg immunoglobulin (anti-HBs) have demonstrated that HBsAg-anti HBs immune complexes can be detected by phagocytosis by polymorphonuclear leucocytes (PMN) (41). In the same study it was reported that phagocytosis of HBsAg-anti HBs immune complexes by PMN is dependent on the antigen/antibody ratio of the immune complexes, and most readily demonstrated if this ratio ranges from 5 to 0.2.

Since conditions of slight antigen excess are known often to result in the formation of pathogenic complexes

with subsequent deposition in renal or other tissues (9, 10), the HBsAg-anti HBs immune complexes demonstrated by phagocytosis by PMN might well correlate with the presence of pathogenic immune complexes.

The purpose of the present study was:

1. to investigate the occurrence of signs and symptoms of systemic disease, possibly related to immune complex deposition in patients with HBsAg-associated hepatitis and healthy HBsAg-carriers.
2. to investigate the occurrence of HBsAg in renal tissue biopsy specimens, studied by the immunofluorescence technique (IF).
3. to study phagocytosis of HBsAg and Ig by PMN both in vitro, and in vivo from the sera of patients with HBsAg-associated hepatitis.

MATERIALS AND METHODS

HBsAg - seropositive subjects, screened for systemic disease.

In 107 HBsAg seropositive subjects with histologically staged liver disease the occurrence of signs and symptoms related to systemic disease, presumably due to circulating immune complexes, were screened, with special reference to joint and skin manifestations and proteinuria. One half of each liver biopsy specimen was routinely prepared for paraffin sections after fixation in either 2% paraformaldehyde or 4% buffered formaldehyde. Sections of 3-4 mu thickness were stained with haematoxylin/azophloxin/safran, elastin-v.Gieson, Periodic acid Schiff with and without diastase treatment, Gomori's reticulin and modificated aldehyde thionin (27) staining techniques. The other half of each biopsy specimen was frozen in liquid nitrogen for IF-study (see below).

The cases of chronic hepatitis were histologically staged according to the criteria proposed by De Groote et al. (8). In the present study, carriers are defined as HBsAg-seropositive subjects without clinical and biochemical signs of liver disease and a liver biopsy specimen showing a normal architecture, no or minimal inflammatory infiltration and clusters of "ground glass" hepatocytes with a blue staining cytoplasm in the aldehyde-thionin staining technique, which cells show cytoplasmic fluorescence in the IF-technique using rabbit-anti HBsAg antiserum (19). Most carriers were identified by routine serological screening of blood donors, followed by taking of a liver biopsy specimen in seropositive subjects. Since any type of hepatitis can be asymptomatic, it was considered justified to perform a percutaneous needle biopsy also in subjects without clinical signs of liver damage and with minimal biochemical changes.

In the course of the hepatitis, skin biopsy specimens were taken from 4 patients for lightmicroscopical examination and IF-study, because of severe itching and urticaria, and from one patient a formalin fixed and paraffin embedded skin, muscle and fascie biopsy specimen was available for lightmicroscopical examination.

Renal tissue specimens.

Lightmicroscopical examination and IF study was performed on 167 consecutive renal biopsy specimens, 11 resected kidneys and 14 resected renal allografts. Tissue for lightmicroscopical examination was routinely prepared for paraffin sections after fixation in 2% paraformaldehyde. Sections of 2 mu thickness were stained with haematoxylin/azophloxin, methenamine silver, elastin v. Gieson and PAS-trichrome staining procedures. Tissue for IF-study was frozen in liquid nitrogen.

PMN phagocytosis test.

The study comprised 35 HBsAg-seropositive patients with histologically characterized liver disease. One additional patient with PAN confirmed by skin and renal biopsy specimens was HBsAg seronegative at the time, the biopsy specimens were taken, but 6 weeks later low titers of HBsAg could be demonstrated.

Peripheral blood PMN were isolated from heparinized venous blood from these patients (direct test) as described elsewhere (41). In addition, normal donor PMN were incubated with serum from these patients (indirect test) and subsequently washed, fixed and frozen as described previously (41).

The sera were allowed to clot during 30 min at 37oC and either freshly incubated with normal donor PMN or stored at -70oC. Some serum samples were tested both fresh and after freezing and thawing several times. In the serum samples used for the indirect PMN-phagocytosis test, the HBsAg and anti-HBs titres were determined by haemagglutination (inhibition) techniques (36), as well as the C1q and C3 levels.

Immunofluorescence procedure.

From the frozen tissue specimens and PMN suspensions 4 mu cryostat sections were cut and prepared for IF study. The indirect immunofluorescence technique was used for the demonstration of IgG, IgM, IgA and C as described previously (40). As second layer horse anti-rabbit antiserum conjugated to fluorescein isothiocyanate (FITC) was used. The presence of HBsAg was determined with the direct technique using rabbit anti-HBsAg antiserum (37), conjugated to FITC and adsorbed with sonificated donor PMN. All antisera were prepared and tested for specificity in the Central Laboratory of the Netherlands Red Cross

Blood Transfusion Service. Slides were read in a Leitz Dialux fluorescence microscope equipped with incident illumination. A Xenon Lamp (Osram XBO. 150 W) was used for excitation.

The percentage of PMN with fluorescent inclusions was determined as previously described (40). Slides with up to 10% of the PMN showing inclusions were scored as +, 10 to 20% was scored as ++ and 1% or less of the PMN showing inclusions was scored as -.

RESULTS

HBsAg-seropositive subjects, screened for systemic disease.

The histological findings, epidemiology and manifestations of systemic disease in the HBsAg-seropositive subjects studied are summarized in table 1. Arthralgia was present in about 20% of the cases, both in male en female patients. However, arthritis with redness, swelling or increased local temperature has not been noted.

The skin biopsy specimens from 4 patients showed minimal perivascular edema and lymphocytic infiltration; by IF study no HBsAg-deposition could be demonstrated, nor Ig and C.

Proteinuria was noted in one case, complicated by severe pyelonephritis, resulting in urosepsis and death. Since permission for autopsy could not be obtained, a histological diagnosis of the renal disease in this patient is not available.

Both the skin and muscle biopsy specimens from the case complicated by PAN showed characteristic fibrinoid necrosis and inflammatory infiltration of the walls of arterioli (Fig.1). IF-studies, performed on sections from the formalin fixed and paraffin embedded skin and muscle biopsy specimens did not demonstrate deposition of either

Table 1

Histological findings, epidemiology and manifestations of systemic disease in 107 HBsAg-seropositive subjects.

	Male	Female
Patients	80	27
Histology		
AcH	15	9
CPH	16	8
CAH	36	4
Cirrh.	15	1
Carrier	10	5
Aspecific	2	1
Epidemiology		
Drug addict	8	2
Homosexual	8	-
Venereal disease	14	-
Tattooing	7	-
Blood transfusion	8	4
Occupational	2	6
Icterus	26	13
Systemic disease		
Urticaria/Rash	1	3
Arthralgia	16	5
Arthritis	-	-
Proteinuria	1	-
PAN	1	-

AcH = acute hepatitis

CPH = chronic persistent hepatitis

CAH = chronic active (aggressive) hepatitis

Cirrh. = cirrhosis

PAN = polyarteritis nodosa

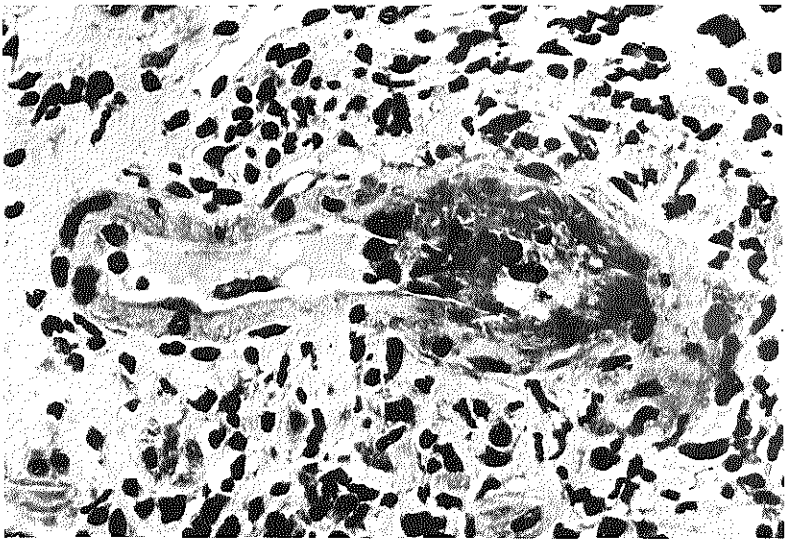

Fig. 1 : Arteriole with segmental fibrinoid necrosis and inflammatory infiltration by lymphocytes. Haematoxylin and azophloxin, X 700.

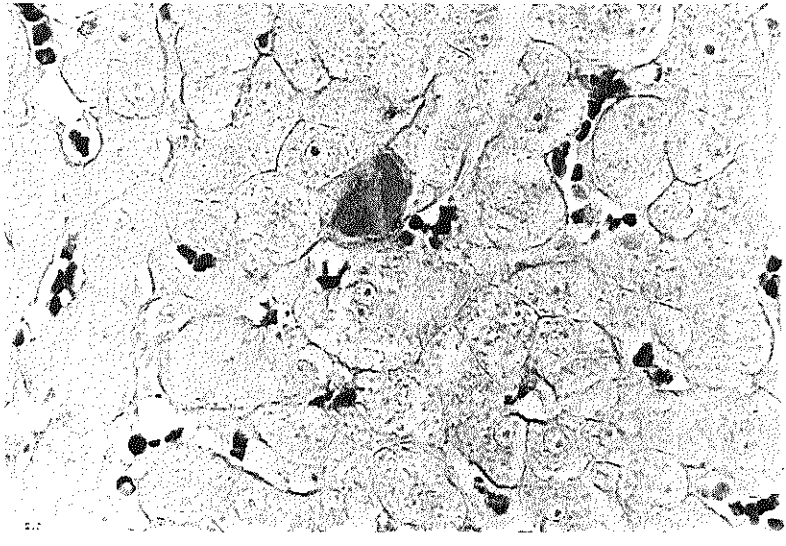

Fig. 2 : Liver tissue with a solitary "ground glass" cell. Aldehyde-thionin, X 750.

ERRATUM

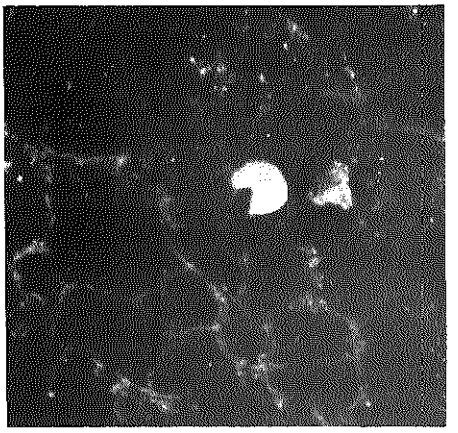

Fig. 3

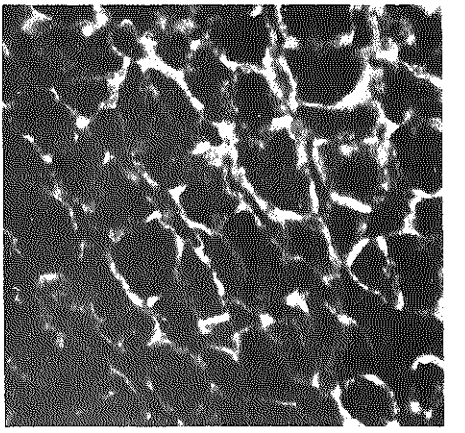

Fig. 4

Fig. 3 : A solitary hepatocyte showing bright cytoplasmic fluorescence by IF study using rabbit anti HBsAg antiserum, X 550.

Fig. 4 : Cell-membrane bound "diffuse" immunofluorescence of hepatocytes, using rabbit anti HBsAg antiserum. X 550.

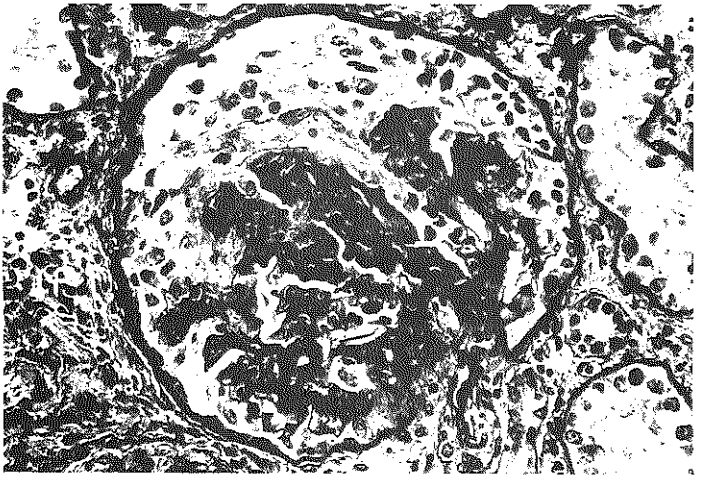

Fig. 5 : Renal glomerulus showing proliferation of the parietal epithelial cells and crescent formation. Methenamine silver, X 360.

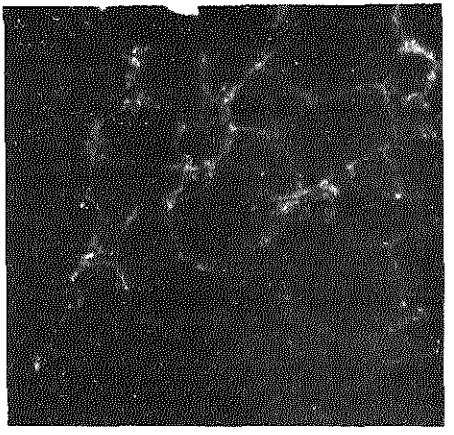

Fig. 3

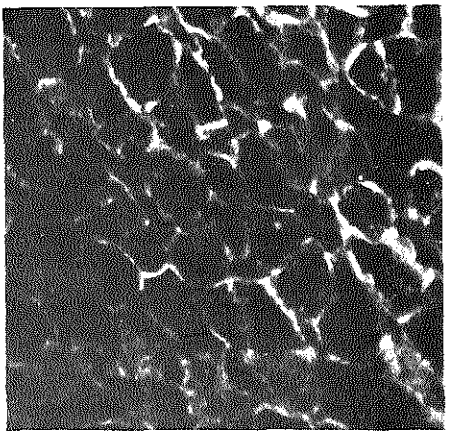

Fig. 4

Fig. 3 : A solitary hepatocyte showing bright cytoplasmic fluorescence by IF study using rabbit anti HBsAg antiserum, X 550.

Fig. 4 : Cell-membrane bound "diffuse" immunofluorescence of hepatocytes, using rabbit anti HBsAg antiserum. X 550.

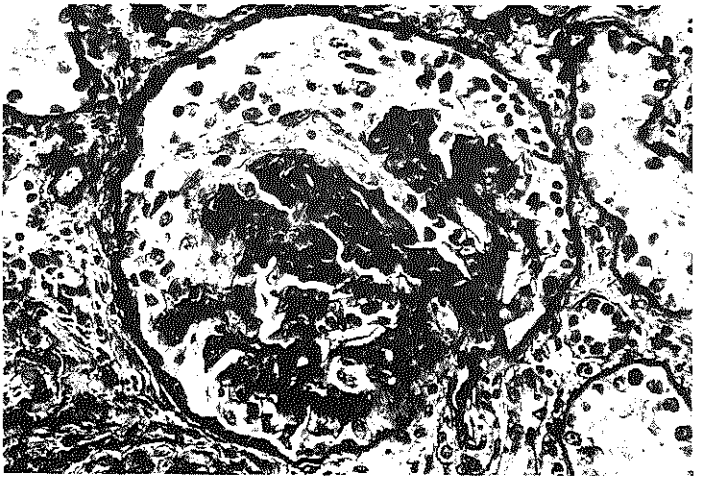

Fig. 5 : Renal glomerulus showing proliferation of the parietal epithelial cells and crescent formation. Methenamine silver, X 360.

HBsAg or Ig and C in the vessel walls. The liver biopsy specimen from this case showed a chronic persistent hepatitis with scattered "ground glass" cells (Fig. 2) showing bright cytoplasmic fluorescence by IF study using anti HBsAg antiserum (Fig. 3).

All cases of CAH showed cell membrane bound fluorescence of the hepatocytes throughout the biopsy specimen ("diffuse pattern" according to Roos et al., (37) using the IF technique with anti HBsAg antiserum (Fig. 4).

Renal tissue specimens.

The histological diagnoses in the kidney biopsy specimens are summarized in tables 2 and 3. Granular deposits of Ig and C were identified along the glomerular basement membrane (GBM) and/or in the mesangium in all cases of endocapillary glomerulonephritis (GN), membranous GN, membranoproliferative GN, local-focal GN and in the case of rapidly progressive GN. Such deposits were also demonstrated in all cases of extracapillary GN, except for two cases of diabetic glomerulopathy without deposits of Ig and C. In one case of diabetic glomerulopathy linear fluorescence of IgG along the GBM was demonstrated, as described by Gallo (13); this case was not interpreted as immune complex GN.

Because of the lightmicroscopical and IF findings it is concluded that in the 192 renal tissue specimens studied 93 cases of GN are caused by deposition of immune complexes.

Three kidney allograft recipients were HBsAg seropositive. Liver biopsy specimens of these patients were not available. In one case the kidney biopsy specimen, taken ½ hour after revascularisation of the allograft, showed bright granular fluorescence along arteriolar vessel walls with antisera against IgM, C and HBsAg.

Table 2

Histological diagnoses on renal tissue from adults (> 15 years)

Tissue	No	Diagnosis	No	Remarks
Nephrectomy	5	End stage kidney	3	
		Pyelonephritis	1	
		Interstitial nephritis	1	Phenacetine abuse
Transplantectomy	14	Rejection	6	
		Circulatory obstruction	5	In one HBsAg-deposition
		Necrosis	2	
		Candida albicans abscesses	1	
Biopsy	53	Recent ischaemic lesion	42	In one HBsAg-deposition along arterioli
½ hour after		Praeexistent ischaemic lesions	7	
transplantation		Praeexistent extracapillary GN	3	
		Praeexistent endocapillary GN	1	
Needle biopsy	74	Ischaemic lesions	12	
		Endocapillary GN	20	
		Extracapillary GN	20	Diabetes mellitus in 3 cases Polyarteritis nodosa in 4 cases
		Membranous GN	7	
		Local - focal GN	6	
		DD: Rapidly progressive GN/PAN	1	
		Interstitial nephritis	3	Phenacetine abuse
		Amyloidosis	2	
		Rejection	3	
		Immune complex GN	55	

Table 3

Histological diagnoses on renal tissue from children (<15 years)

Tissue	No	Diagnosis	No	Remarks
Nephrectomy	6	Membranoproliferative GN	2	
		Bleeding after biopsy procedure	1	
		Focal glomerulosclerosis	1	
		End stage kidney	2	
Biopsy ½ hour after transplantation	2	Recent ischaemic lesion	2	
Needle biopsy	38	Endocapillary GN	20	
		Extracapillary GN	12	
		Local-focal GN	3	
		Membranous GN	1	
		Amyloidosis	1	
		Interstitial fibrosis	1	Leucaemia in remission
		Immune complex GN	38	

However, at autopsy, neither in the transplanted kidney, nor in the own kidneys of the patient could HBsAg, Ig or C be detected by IF, 3 weeks after transplantation. The patient died of bronchopneumonia with lung abscesses. Two cases showed only slight edema and shedding of tubular epithelium in the ½ hour biopsy specimen. However, three weeks after transplantation transplantectomy was performed in one case because of ischaemic necrosis of the kidney, caused by arterial thrombotic obstruction. The resected kidney showed granular deposits of HBsAg, IgM and C along vessel walls and glomerular capillary loops. The presence of HBsAg could not be demonstrated in any of the other kidney biopsy specimens studied.

In one of the four cases of PAN, histologically confirmed by a renal and skin biopsy specimen, HBsAg could not be demonstrated at the time the biopsy specimens were taken, but 6 weeks later low titers of HBsAg were present. A liver biopsy specimen of this case was not available. The renal biopsy specimen showed a focal extra-capillary proliferative glomerulonephritis with crescent formation (Fig. 5).

PMN phagocytosis test.

The results of the PMN phagocytosis test, in relation to the histological diagnoses on the liver biopsy specimens and the serological findings concerning complement levels and titres of HBsAg and anti HBs are summarized in table 4. The presence of both Ig and C in the PMN was interpreted as suggestive of immune complexes in the serum tested, and was demonstrated in 4 out of 16 cases of CAH and in two cases of PAN. Evidence of HBsAg containing immune complexes was obtained in one additional case of CAH by demonstration of HBsAg in association with Ig and C in the PMN.

The liver biopsy specimen in one of the cases with PAN showed CPH. The other case of PAN was confirmed by a

Table 4

Histological and serological findings in 35 HBsAg seropositive patients and in one case of PAN

				PMN phagocytosis test								serum titer	
				Direct			Indirect						
Pat.	m/f	age	Histol.diagnosis	HBsAg	Ig	C	HBsAg	Ig	C	C1q	C3	HBsAg	anti HBs
1	m		CAH	-	-	-	-	-	-			+	
2	m	38	Severe CAH	-	-	-	-	-	-			+	
3	f	24	AcH	-	-	-	-	-	-			+	
4	m	38	CPH/slight CAH		N.D.		-	-	-			+	
5	f	28	AcH + necrosis		N.D.		-	-	-			+	
6	m	65	Cirrh.		N.D.		-	-	-			+	
7	m	40	AcH		N.D.		-	-	-			+	
8	m	44	CAH + fibrosis		N.D.		-	-	-			+	
9	m	60	AcH		N.D.		-	-	-	103	95	12,800	-
10	m	40	Severe CAH + cirrh.		N.D.		++	++	++	138	131	1,000	-
11	m	21	CAH + cirrh.		N.D.		-	-	-	99	109	2,000	-
12	f	26	AcH		N.D.		-	-	-	82	89	3,200	-
13	m	54	CAH + cirrh.		N.D.		-	+	+			+	
14	m	50	CAH + cirrh.		N.D.		-	-	-	95	68	6,400	-
15	m	26	Healthy carrier		N.D.		-	-	-	59	71	+	
16	m	48	CAH + cirrh.		N.D.		-	-	-			+	
17	m	43	AcH		N.D.		-	-	-			+	

18	m	23	AcH		N.D.		-	-	-			+		
19	m	32	No liver tissue	-	+	+	-	+	+	118	41	-	-	
			available	-	+	+	-	+	+	102	56	64	-	
20	m	73	CPH		N.D.		-	+	+	79	68	51,200	-	
21	m	53	CAH + cirrh.	-	+	+	-	+	+	136	109	3,200	128	
22	m	32	AcH	-	-	-		N.D.				+		
23	m	20	CPH	-	-	-	-	-	-	82	97	1,000	-	
24	m	57	CPH	-	-	-	-	-	-	122	89	2,000	64	
25	m	30	CAH	-	-	-	-	-	-	89	51	3,200	16	
26	m	29	CPH	-	-	-	-	-	-	108	80	12,800	-	
27	f	76	CAH + cirrh.	-	-	-	-	-	-	122	119	6,400	-	
28	f	19	CAH (severe)	-	-	-	-	+	+	79	95	+		
29	m	28	AcH	-	-	-	-	-	-	↓↓	39	8	-	
30	m	26	CPH	-	-	-	-	-	-	122	97	3,200	-	
31	f	33	CPH	-	-	-	-	-	-			+	-	
32	m	33	CAH	-	-	-	-	-	-	99	68	3,200	-	
33	m	39	Healthy carrier	-	-	-	-	-	-	68	56	32	-	
34	m	63	CAH + cirrh.	-	-	-	-	+	+	82	80	12,800	-	
35	m	34	CAH	-	+	-	-	-	-	108	68	3,200	-	
36	m	30	AcH	-	-	-	-	-	-	56	165	64	-	
				-	-	-	-	-	-	65	126	16	-	
				-	-	-	-	-	-	39	87	6	-	
				-	-	-	-	-	-	42	124	-	-	
				-	-	-	-	-	-	34	79	-	-	
				-	-	-	-	-	-	59	99	-	-	

renal (Fig. 5) and skin biopsy specimen, 6 weeks before HBsAg could be demonstrated in the serum. A liver biopsy specimen of this patient was not available.
None of the patients had signs or symptoms of systemic disease at the moment of blood sampling, except for the two cases of PAN and patient (36), who had arthralgia at the time that two of the six weekly obtained blood samples were taken. However, five patients had a history of arthralgia in the prodromal phase of hepatitis some weeks earlier, and a rash had been noted in one case.

DISCUSSION

The striking male preponderance of HBsAg-associated hepatitis in this study confirms the findings of several authors (18, 42) and is caused predominantly by the frequent occurrence of CAH in men, often complicated by cirrhosis, as has also been reported by Klatskin (23).

Some of the high risk groups (i.e. hospital and laboratory personel, drug addicts, tattooed subjects) are directly related to an increased chance of parenteral infection by contaminated blood. The striking number of male homosexual subjects and patients with venereal disease as found in our series, has also been noted in other studies (12, 22). Microtrauma at sexual intercourse has been suggested as a possible cause of the increased risk of hepatitis B infection in these subjects (43).

Several studies (15, 25) have drawn attention to extrahepatic manifestations of viral hepatitis and the role of circulating immune complexes in the pathogenesis of these syndromes. Gocke (15) recognized three types of syndromes: first a serum sickness like prodrome of acute hepatitis consisting of urticaria, rash, polyarthralgia and sometimes arthritis in 15 - 20 % of patients. Secondly, he noted an incidence of 30-40% of HBsAg-antigenaemia in

patients with PAN, and thirdly, an immune complex type of glomerulonephritis, usually in association with chronic active hepatitis.

The frequency of joint manifestations in hepatitis B in our study (21 out of 107 cases) confirms the findings of Gocke (15) and Mosley and Galambos (30) who reported an incidence of 20.6% of joint symptoms in hepatitis after specifically asking for it. However, arthritis with evidence of redness, swelling and/or increased local temperature was not present in any of the patients studied. In a review of the literature, Alarcon and Townes (1) reported that arthritis is a relatively uncommon complication of hepatitis while arthralgia occurs much more frequently. These authors reported no sex differences for the incidence of arthralgia in patients with hepatitis, which is confirmed in our study (16 out of 80 male patients and 5 out of 27 female patients). In contrast, they reported a slight predominance of female patients in cases complicated by arthritis (3:2). However, Kohler (25) reported that joint and skin symptoms occurred significantly more frequently in female compared to male hepatitis B patients (48 and 15 % resp.).

In most of our cases complicated by joint and skin manifestations, the symptoms occurred during the prodromal and/or early acute phase of hepatitis, as was also reported by Gocke (15), except for one case with urticaria occurring shortly before serological tests indicated the presence of anti HBs instead of HBsAg.

Although the most attractive hypothesis for the pathogenesis of skin and joint manifestations is the deposition of circulating immune complexes, followed by an inflammatory response, histological and IF evidence of such complex deposition in the tissues is lacking. In our study, 4 skin biopsy specimens, taken because of severe itching and urticaria, did not show deposition of HBsAg or Ig, as demonstrated by IF. Synovial biopsy

specimens were not available.

Except for one synovial biopsy specimen examined by lightmicroscopical and IF techniques (11) without Ig and C deposition (HBsAg not tested) and one skin biopsy specimen examined only by light microscopy (48) no histological data are available in the literature studied.

Evidence of circulating immune complexes in the prodromal syndrome of arthralgia and rash of acute hepatitis is mainly based on complement component studies (2, 28, 34, 48). However, involvement of HBsAg in complex formation, resulting in low levels of C-components, was not demonstrated. Onion et al. (34) reported that in no instance HBsAg and anti HBs were detected together in the same serum samples from 3 patients with active arthritis, although all patients showed low CH50-values.
None of the patients studied by Alpert et al. (2) had demonstrable anti HBs in their serum during active arthritis, nor in convalescence or remission, and it was stressed that serum HBsAg titres were high. However, because of the results of the study of experimental serum sickness, indicating that conditions of slight antigen excess may result in pathogenic immune complexes (6, 9), low serum titres of HBsAg might be expected if pathogenic circulating immune complexes are present.

Therefore, we conclude that complement component studies have provided circumstantial evidence of the presence of circulating immune complexes, although involvement of HBsAg has not been demonstrated. The possibility of other hepatitis B associated antigens (e.g. hepatitis B core antigen: HBcAg) being responsible for the formation of pathogenic complexes should be considered. The appearance of anti HBcAg antibodies (anti HBc) at the beginning of clinical signs of acute hepatitis (21) coincides well with the expected sequence of serological events if HBcAg-anti HBc immune complexes are responsible for skin and joint manifestations in the prodromal phase

of acute hepatitis.

Analysis of cryoprecipitable proteins in serum from hepatitis B patients has provided evidence of HBsAg containing circulating immune complexes (29, 47). However, the results obtained from cryoprotein analysis in relation to clinical manifestations of systemic disease are controversial. Wands et al. (47) reported both HBsAg and anti HBs in the cryoprecipitate in one out of three patients with acute hepatitis complicated by arthritis, while both antigen and antibody disappeared from serum and cryoprecipitate when the arthritic symptoms subsided. McIntosh et al. (29) reported the highest amounts of cryoprecipitable material in patients with acute hepatitis (a relation to systemic disease is not reported), but stressed that systemic disease occurred predominantly in patients with chronic hepatitis. These authors demonstrated both antigen and antibody in the cryoprecipitates from patients with chronic hepatitis without vasculitis, while Sergent et al. (38) in a study concerning longterm observations in hepatitis B patients with PAN, were not able to demonstrate cryoglobulins in any of the patients studied.

Therefore we conclude that cryoprotein analysis may contribute to the detection of circulating immune complexes, although the reports published so far indicate that their presence does not show significant correlation with systemic disease in hepatitis B.

A causal relationship between hepatitis B and PAN could not be proven in the one case of PAN in association with chronic persistent hepatitis in our study. Deposition of HBsAg-containing immune complexes in the vessel walls could not be investigated, since frozen biopsy specimens were not available for IF-study. The results of the indirect PMN phagocytosis test were identical with those in a case of PAN, in which HBsAg was first demonstrated in low titres 6 weeks after the histological confirmation

of arteritis and glomerulonephritis. It is suggested that circulating immune complexes without detectable HBsAg were present in the serum during active vasculitis in these cases.

Histological and IF data on 19 cases of PAN in association with HBsAg were reported by several authors (3, 14, 17, 20, 32) In two cases reported by Gocke (17) and in 6 of Nowoslawski's cases (32) deposits of both HBsAg and Ig in the vessel walls were demonstrated. Although HBsAg, without Ig and C was demonstrated in vessel walls in the cases of Gerber et al. (14) and Baker et al. (3), these deposits might be secondary to tissue damage, as was concluded by Gerber et al. (14). Convincing histological evidence of involvement of HBsAg in the pathogenesis of PAN was also lacking in the 5 cases of Heathcote et al. (20) and in 4 of Gocke's cases (17). No IF-studies including anti-HBsAg antiserum were performed in the cases of Prince and Trepo (35), Kohler (25), Sergent et al. (38) and Duffy et al. (11) and histological data concerning the cases of Trepo et al. (46) are not reported. In contrast to the joint and skin manifestations of the serum sickness like prodrome of acute hepatitis, data compiled from the studies of Trepo et al. (45), Heathcote et al. (20), Gerber et al., (14), Baker et al., (3), Nowoslawski et al. (32), Sergent et al. (38) and Duffy et al. (11) indicate that the frequency of hepatitis B associated PAN is very low and that no significant relation to any of the histological types of hepatitis can be recognized.

Glomerulonephritis was not present in the 107 cases of hepatitis B studied. Although proteinuria was noted in one case, this could well be explained by an exacerbation of chronic pyelonephritis, ultimately resulting in urosepsis and death. On the other hand, in IF-studies on 93 cases of glomerulonephritis, presumably caused by deposition of immune complexes, HBsAg could not be

demonstrated in any of the biopsy specimens. The findings of HBsAg, IgM and C deposits in a resected renal allograft showing extensive ischaemic necrosis confirms the findings of Gerber et al. (14), who reported HBsAg deposition in a renal infarction and suggested that these deposits were secondary to tissue damage. We have no satisfactory explanation for the deposition of HBsAg, IgM and C along arteriolar vessel walls in the renal biopsy specimen, taken half an hour after revascularisation of the allograft, in one out of three HBsAg seropositive kidney transplant recipients. Immune complex glomerulonephritis could not be confirmed at autopsy 3 weeks after transplantation, and at that time HBsAg could not be detected in the renal allograft.

In the literature studied, 10 cases of glomerulonephritis with HBsAg containing glomerular immune complex deposits are reported in association with histologically typed hepatitis. (7, 24, 26, 31, 32). In addition, 21 cases of glomerulonephritis with HBsAg containing deposits are reported without histological data concerning hepatic involvement (4, 29). In most cases (8/10) the diagnosis on the liver biopsy specimen was chronic aggressive hepatitis. Although in Myers' case (31) the liver biopsy specimen was interpreted as chronic persistent hepatitis, neither the published photomicrograph nor the histologic description in the text are consistent with this diagnosis and it is most likely that chronic aggressive hepatitis with cirrhosis is the correct diagnosis. Thus, in contrast to the findings in hepatitis B associated PAN, glomerulonephritis occurs predominantly in association with CAH. Furthermore, HBsAg-containing immune complex deposits have been demonstrated in most cases of hepatitis B associated glomerulonephritis, as opposed to many cases of hepatitis B associated PAN lacking IF evidence of HBsAg deposition . The startling prevalence of HBsAg in children with immune complex glomerulonephritis (18/32)

as reported by Brzosko et al. (4) was not confirmed in our study (o/38) nor by Knieser et al. (24) who mentioned a prevalence of 2/145 cases (not specified for children and adults).

The finding of Ig and C phagocytosis by PMN almost exclusively from sera of patients with CAH supports the observation of Gocke (15) that in this type of hepatitis B the risk of complicating systemic disease (i.e. glomerulonephritis) is highest. Only in two cases PMN inclusions were demonstrated unassociated with CAH, and both concerned cases of PAN with active vasculitis.

Since IF studies of liver tissue have demonstrated the presence of HBsAg along the cellular membrane of hepatocytes ("diffuse pattern" according to Roos et al. (39) in CAH, it is conceivable that this membrane bound foreign antigen induces the host defense mechanism to a chronic breakdown of liver cells, ultimately resulting in fibrosis or cirrhosis. In addition, shedding of cell membrane bound antigen-antibody complexes may occur, and this is probably reflected by phagocytosis by PMN. When elimination of the "shedded" immune complexes is insufficient, deposition in vessel walls or glomeruli with subsequent tissue damage may result. This mechanism may be independent of the production of HBsAg, and therefore also of serum titres of HBsAg. This is confirmed by the fact that a significant relation between HBsAg serum titres on one hand and either the histological type of hepatitis or the presence of immune complexes as demonstrated by the PMN phagocytosis test on the other hand, could not be demonstrated.

Low complement levels were demonstrated only in one case of acute hepatitis, and these might well be due to depressed synthesis caused by liver damage.

The simultaneous occurrence of HBsAg and anti HBs was demonstrated in 3 serum samples. In one case this finding coincided with the presence of immune complexes

as demonstrated by the PMN phagocytosis test. In the other two cases apparently no phagocytizable immune complexes were present and in none of these cases systemic manifestations attributable to circulating immune complexes were present.

As outlined above, circulating immune complexes may well be due to shedding of primarily tissue-bound antigen-antibody complexes, rather than being the result of interaction of free circulating antigen and antibody. This implies that the simultaneous detection of HBsAg and anti HBs in serum samples is probably not a usefull parameter for the presence of circulating immune complexes, as indeed is confirmed by our finding that in the only case in which the involvement of HBsAg was demonstrated in the PMN phagocytosis test, no detectable anti HBs was present in the serum.

Since in most cases no antigen was identified in the PMN, the possibility of an artifact being responsible for the in vitro phagocytosis of Ig and C should be considered. This phenomenon might be caused by aggregation of Ig, induced by freezing and thawing of the serum samples. Therefore, the indirect PMN phagocytosis test was performed both with fresh serum from patients and controls and after repeated freezing and thawing of the same samples. In no case was phagocytosis of Ig or C demonstrated from the frozen and thawed samples only. This finding confirms the results of Cats et al. (5), who also reported that freezing and thawing of serum samples did not alter the results of the indirect PMN phagocytosis test.

Conclusions:

1. This study confirms an incidence of arthralgia of 20% in the prodromal or early acute phase of hepatitis B, as reported in the literature. It is suggested that hepatitis B associated antigens, other than HBsAg are involved in

the pathogenesis of joint symptoms. Evidence of HBsAg-containing immune complexes in the pathogenesis of these joint symptoms is lacking.

2. A causal relationship of HBsAg and vasculitis has only been demonstrated in a minority of well documented cases of PAN. There is no significant relation between the histological type of hepatitis and the occurrence of PAN. The presence of circulating immune complexes in two cases of hepatitis B associated PAN, studied during active vasculitis, has been demonstrated by the PMN phagocytosis test, although involvement of HBsAg could not be demonstrated in these cases.

3. A causal relationship of HBsAg and glomerulonephritis has been well documented in the literature. In addition, the concomitant histological type of hepatitis is predominantly CAH.

4. The results of the PMN phagocytosis test, indicating the presence of circulating immune complexes in 5 out of 16 cases of CAH, fit in well with a hypothesis concerning the origin of circulating HBsAg-anti HBs immune complexes in hepatitis B based on histological and IF findings in liver biopsy specimens and serological studies of the same cases, and on an analysis of data compiled from the literature.

REFERENCES

1. Alarcon, G.S. and Townes, A.S. Arthritis in viral hepatitis. Report of two cases and review of the literature. Hopkins Med.J. 132, 1. 1973.
2. Alpert, E., Isselbacher, K.J. and Schur, P.H. The pathogenesis of arthritis associated with viral hepatitis. Complement component studies. N.Engl.J.Med. 285: 185. 1971.
3. Baker, A.L., Kaplan, M.M., Benz, W.C., Sidel, J.S. and Wolfe, H.J. Polyarteritis associated with Australia-antigen positive hepatitis. Gastroenterology 62: 105. 1972.
4. Brzosko, W.J., Krawczynski, K., Nazarewicz, T., Morzycka, M. and Nowoslawski, A. Glomerulonefritis associated with hepatitis B surface antigen immune complexes in children. Lancet 2: 477. 1974.
5. Cats, A., Lafeber, G.J.M. and Klein, F. Immunoglobulin phagocytosis by granulocytes from sera and synovial fluids in various rheumatoid and non rheumatoid diseases. Ann.Rheum. Dis. 34, 146. 1975.
6. Cochrane, C.G. and Koffler, D. Immune complex disease in experimental animals and man. Immunol. 16, 185. 1973.
7. Combes, B., Stastny, P., Shorey, J., Eigenbrodt, E.H., Barrera, A., Hull, A.R., Carter, N.W. Glomerulo-nefritis with deposition of Australia antigen-antibody complexes in glomerular basement membrane. Lancet 2, 234. 1971.
8. De Groote, J., Desmet, V.J., Bedigle, P., Korb, B., Popper, H., Poulsen, H., Scheuer, P.J., Schmid, M., Thaler, H. and Uehlinger, E. A classification of chronic hepatitis. Lancet 2, 626., 1968.
9. Dixon, F.J., Vazquez, J.J., Weigle, W.O. and Cochrane, C.G. The pathogenesis of serum sickness. Arch. Pathol. 65, 18, 1958.
10. Dixon, F.J. Mechanisms of immunologic injury. In: Immunobiology. Editors: Good, R.A. and Fisher, D.W., Sinauer Associates, Inc., Stamford, Connecticut. 1971.
11. Duffy, J., Lidsky, M.D., Sharp, J.T., Davis, J.S.. Person, D.A., Hollinger, F.B. and Min, K.W. Poly-arthritis, polyarteritis and hepatitis B. Medicine 55, 19. 1976.
12. Fulford, K.W.M., Dane, D.S., Catterall, R.D., Woof, R. and Denning, J.V. Australia antigen and antibody among patients attending a clinic for sexually transmitted diseases. Lancet 1, 1470. 1973.
13. Gallo. G.R. Elution studies in kidneys with linear deposition of immunoglobulin in glomeruli. Am.J. Pathol. 61, 377. 1970.
14. Gerber, M.A., Brodin, A., Steinberg, D., Vernace, S., Yang, C.P. and Paronetto, F. Periarteritis nodosa, Australia antigen and lymphatic Leucaemia. N.Eng.J.Med. 286, 14. 1972.

15.Gocke, D.J. Extrahepatic manifestations of viral hepatitis. Am.J.Med. Sci. 270, 49. 1975.
16.Gocke, D.J. , Morgan, C., Lockshin, M., Hsu, K., Bombardieri S., Christian, C.L. Association between polyarteritis and Australia antigen. Lancet 2, 1149-1970.
17.Gocke, D.J., Hsu, K., Morgan, C., Bombardieri, S., Lockshin, M., Christian, C.L. Vasculitis in association with Australia antigen. J. Exp. Med. 134, 330S. 1971.
18.Goodman, M., Wainright, R.L., Weir, H.F. and Gall. J.C. A sex difference in the carrier state of Australia (hepatitis associated) antigen. Pediatricts 48, 907. 1971.
19.Hadziyannis, S., Gerber, M.A., Vissoulis, Ch. and Popper, H. Cytoplasmic hepatitis B antigen in "Ground-Glass" hepatocytes of carriers. Arch. Pathol. 96, 327. 1973.
20.Heathcote, E.J.L., Dudley, F.J. and Sherlock, S. The association of polyarteritis and Australia antigen. Digestion, 6, 280. (abstr.) 1972.
21.Hoofnagle, J.H., Gerety, R.J. and Barker, L.F. Antibody to hepatitis B-virus core in man. Lancet 2, 869. 1973.
22.Jeffries, D.J., James, W.H., Jefferiss, F.J.G., McLeod, K.G. and Willcox, R.R. Australia (Hepatitis associated) antigen in patients attending a venereal disease clinic. Brit. Med.J. 2, 455. 1973.
23.Klatskin, G. Persistent HB antigenemia: associated clinical manifestations and hepatic lesions. Am.J.Med.Sci. 270, 33. 1975.
24.Knieser, M.R., Jenis, E.H. Lowenthal., Bancroft, W.H., Burns, W., Shalhoub, R. Pathogenesis of renal disease associated with viral hepatitis. Arch. Pathol. 97, 193. 1974.
25.Kohler, P.F. Clinical immune complex disease. Manifestations in systemic lupus erythematosus and hepatitis B virus infection. Medicine 52, 419. 1973.
26.Kohler, P.F., Cronin, R.E., Hammond, W.S., Olin, D., Carr, Carr, R.I. Chronic membranous glomerulonephritis caused by hepatitis B antigen-antibody immune complexes. Ann. Int. Med. 81, 448. 1974
27.Luna, L.G., Editor. Manual of histologic staining methods of the Armed Forces Institute of Pathology Third Edition. McGraw-Hill. New York, Toronto, London, Sydney. 1968. p. 210.
28.McCarty, D.J. and Ormiste, V. Arthritis and HBAg-positive hepatitis. Arch. Intern. Med. 132, 264.1973
29.McIntosh, R.M., Koss, M.N. and Gocke, D.J. The nature and incidence of cryoproteins in hepatitis B antigen (HBsAg) positive patients. Quart.J.Med. 45 (69) 23. 1976.
30.Mosley, J. and Galambos, J. Viral hepatitis. In: Diseases of the Liver. Edited by Leon Schiff, J.B. Lippincot Co., Philadelphia, Toronto. 1969.

p. 448.
31.Myers, B.D., Griffel, B., Naveh, D., Jankielowitz, T., Klajman, A. Membrano-proliferative glomerulonephritis associated with persistent viral hepatitis. Am. J. Clin. Pathol. 60, 222. 1973.
32.Nowoslawski, A., Krawczynski, K., Brzosko, W.J., and Madalinski, K. Tissue localisation of Australia antigen immune complexes in acute and chronic hepatitis and liver cirrhosis. Am. J. Pathol. 68, 31. 1972
33.Nydegger, U.E., Lambert, P.H., Gerber, H. and Miescher, P.A. Circulating immune complexes in the serum in systemic lupus erythematosus and in carriers of hepatitis B antigen. Quantitation by binding to radiolabeled C1q. J. Clin. Invest. 54, 297. 1974.
34.Onion, D.K., Crumpacker, C.S. and Gilliland, B.C. Arthritis of hepatitis associated with Australia antigen. Ann. Intern. Med. 75, 29. 1971.
35.Prince, A.M. and Trepo, C. Role of immune complexes involving SH antigen in pathogenesis of chronic active hepatitis and polyarteritis nodosa. Lancet 1, 1309. 1971.
36.Reesink, H.W., Duimel, W.J.M. and Brummelhuis, H.G.J. Standardization and modification of the haemagglutination (inhibition) techniques for the determination of HBAg and anti-HBAg antibodies. Vox Sang. 24, 528. 1973.
37.Roos, C.M., Feltkamp-Vroom, Th.M., and Helder, A.W. The localization of hepatitis B antigen and immunoglobulin G in liver tissue; an immunofluorescence, light and electron microscopic study. J.Pathol. 118, 1. 1976.
38.Sergent, J.S., Lockshin, M.D., Christian, C.L. and Gocke, D.J. Vasculitis with hepatitis B antigenemia: Long-term observations in nine patients.Medicine 55, 1. 1976.
39.Shulman, N.R. and Barker, L.F. Virus-like antigen, antibody and antigen-antibody complexes in hepatitis measured by complement fixation. Science 165, 304. 1969.
40.Steffelaar, J.W., de Graaff-Reitsma, C.B. and Feltkamp-Vroom, Th.M. Immune complex detection by immunofluorescence on peripheral blood polymorphonuclear leucocytes. Clin. Exp. Immunol. 23, 272.1976.
41.Steffelaar, J.W., ten Kate, F.J.W., Nap, M., Swaak, A.J.G., de Graaff-Reitsma, C.B., v. Elven, E.H. and Feltkamp-Vroom, Th. M. Immune complex detection by immunofluorescence on polymorphonuclear leucocytes. Clin. exp. Immunol.: accepted for publication.
42.Szmuness. W., Hirsch, R.L. Prince, A.M., Levine, R.W. Harley, E.J. and Ikram, H. Hepatitis B surface antigen in blood donors: Further observations. J.Infect. Dis. 131, 111. 1975.
43.Szmuness, W., Much, M.I., Prince, A.M. Hoofnagle, J.H., Cherubin, C.E., Harley, E.J. and Block, G.H.

On the role of sexual behavior in the spread of hepatitis B infection. Ann.Intern.Med. 83, 489. 1975.
44.Theofilopoulos, A.N. Wilson, C.B. and Dixon, F.J. The Raji cell radio immune assay for detecting immune complexes in human sera. J. Clin. Invest. 57, 169. 1976.
45.Trepo, C., Thivolet, J. and Lambert, R. Four cases of periarteritis nodosa associated with persistent Australia antigen. Digestion 5, 100.1972.
46.Trepo, C.G., Zuckerman, A.J., Bird, R.C. and Prince, A.P. The role of circulating hepatitis B antigen/antibody immune complexes in the pathogenesis of vascular and hepatic manifestations in polyarteritis nodosa. J. Clin. Path. 27, 863. 1974.
47.Wands, J.R., Mann, E., Alpert, E. and Isselbacher, K.J. The pathogenesis of arthritis associated with acute hepatitis B surface antigen-positive hepatitis Complement activation and characterization of circulating immune complexes. J. Clin. Invest. 55. 930. 1975.
48.Wenzel. R.P., McCormick, D.P., Busch, H.J., Beam. W.E. Arthritis and viral hepatitis. A patient with transient serum hepatitis-associated antigen, skin nodules, rash and low serum complement. Arch. Intern. Med. 130. 770. 1972.

CHAPTER VI: DISCUSSION

In this thesis some experiments and clinical, histological and immunofluorescence studies are reported, which were designed with the purpose to develop a technique which enables to monitor diseases, mediated by circulating immune complexes. In order to be of clinical interest, the technique should meet the following requirements:

1. The results of the test should correlate with clinical signs and symptoms of immune complex mediated disease, i.e. the test should discriminate potentially pathogenic from "harmless" complexes.
2. The test should include the possibility to detect the antigen involved in immune complex formation.
3. Preferentially, the laboratory outfit needed should be relatively simple (e.g. no radiolabeled reagents, no need for an ultracentrifuge or an electron microscope), and to perform the test, only commercially available reagents should be necessary.

An immune complex detection technique, which meets all these requirements is not available. As outlined in chapter I, many of these techniques need the use of radiolabeled reagents and/or an ultracentrifuge, e.g. the techniques described by Farr (1958), Nydegger et al. (1974) and Sobel et al. (1975). Tha Raji cell radio immuno assay (Theofilopoulos et al., 1976) requires in addition the availability of a lymphoblastoid cell line derived from a Burkitt lymphoma. Published reports concerning the correlation of the results of an immune complex detection technique and clinical signs and symptoms of immune complex mediated disease are scarce, and several of these studies do not show a significant correlation between the complex-mediated disease and the results of the techniques used (Prince and Trepo, 1971; Gerber et al., 1972; Wands et al., 1975; Sergent et al., 1976; McIntosh

et al., 1976).

Observations concerning phagocytosis of Ig and C by PMN, both under experimental conditions in vitro (Parker and Schmid, 1962) and in vivo (Cochrane et al., 1959), and also demonstrated in the studies by Rawson et al. (1965), Vaughan et al. (1968) and Cats et al. (1975), established the basis for the assay for immune complex detection, presented in this thesis, since the inclusions of Ig and C in PMN, reported in the above mentioned studies, probably represented phagocytized immune complexes.

Therefore, a technique was developed to screen large numbers of PMN for the presence of Ig and C inclusions. At first, PMN from the blood of patients were studied (direct test) and later on studies of PMN from the blood of normal donors, after incubation with the serum to be tested for the presence of immune complexes (indirect test) were included. The isolation procedure for PMN, reported in chapter II, followed by freezing of the PMN-suspensions and subsequent cutting of 4 mu sections in a cryostat proved to be useful in order to obtain preparations that are suitable for IF-studies of these cells. The main advantages of this technique are that firstly a large number of sections can be cut from a rather small sample and that secondly sections can be cut at any desired moment after preparation of the frozen suspensions. Nuclear counterstaining with ethidium bromide allows easy identification of the nuclei (Udenfriend, 1969), using the same excitation light as for FITC fluorescence. This quick and simple method has proved to be very useful for determining the number of PMN that do not show FITC fluorescence. The results reported in this chapter, indicated the presence of Ig and C in up to 80% of the PMN isolated from defibrinated blood from SLE-patients.

Since preliminary results indicated that PMN, isolated from heparinized blood contained less and smaller Ig and C

inclusions than PMN from defibrinated blood from the same patients, some of the experiments described in chapter III were designed to investigate the influence of several anticoagulants and defibrination on the percentage of PMN with Ig and C inclusions. These studies demonstrated a loss of about 20% of PMN, harvested from defibrinated blood, as compared to heparinized or EDTA blood. Cellular destruction by glass beads is most probably responsible for at least part of the loss, since heparinized blood samples which were shaken with glass beads as in the defibrination procedure, also showed a loss of about 20% of the PMN. The high percentages of PMN with Ig and C inclusions, as demonstrated in defibrinated samples, are attributed partly to in vitro phagocytosis of immune complexes, which resulted from the release of nuclear antigens, caused by cellular destruction, in the presence of ANA. In heparinized blood samples from SLE-patients, blocking of in vitro phagocytosis by the addition of MIAA did not result in a reduction of the number of PMN with Ig and C inclusions, as compared with PMN from samples without MIAA. Therefore it is most likely that these inclusions represent in vivo phagocytosis of immune complexes. In this chapter, the studies of Cats et al. (1975) were confirmed, who reported that incubation of normal donor PMN with serum from SLE-patients resulted in phagocytosis of Ig and C (indirect test).

Because of the possible pathogenetic role of immune complexes in systemic manifestations of hepatitis B (Gocke, 1975), in vitro studies were performed with artificially prepared HBsAg-anti HBs immune complexes. In addition, the influence of the antigen/antibody ratio on the in vitro phagocytosis of these complexes was tested. The results of these studies indicated that in PMN, incubated with immune complexes with an antigen/antibody ratio lying between 5 and 0.2, both the antigen (HBsAg) and antibody could be demonstrated.

Since it may be postulated that in human hepatitis B, as in experimental serum sickness, approximate balance of HBsAg and anti HBs results in pathogenic immune complexes, the indirect PMN phagocytosis test should be able to detect potentially pathogenic HBsAg-anti HBs immune complexes.

Several authors reported techniques which detected in up to 95% of hepatitis B patients at some stage of the disease the occurrence of HBsAg-containing immune complexes in the serum (Shulman and Barker, 1969; Prince and Trepo, 1971; Nydegger et al., 1974; McIntosh et al., 1976; Theofilopoulos et al., 1976). However, only a small minority of the immune complexes detected by the techniques used can be relevant as far as immune complex deposition, resulting in clinical manifest systemic disease, is concerned, since the prevalence of HBsAg-associated hepatitis in western countries is about 1.90 per 1,000 (e.g. Szmuness et al., 1975) and the number of well documented cases of systemic disease, caused by HBsAg containing immune complexes published so far, is only about 45.

Therefore, we studied the incidence of signs and symptoms of systemic disease, possibly related to immune complex deposition in patients with histologically staged HBsAg-associated hepatitis, as well as the occurrence of HBsAg in a series of renal tissue specimens. In addition, the PMN phagocytosis test was performed in 35 patients with histologically staged hepatitis B and in one patient with PAN. The results of these studies, reported in chapter V, indicate that arthralgia mainly occurs in the prodromal or early acute phase of hepatitis B, with an incidence of about 20%. This is also reported by Mosley and Galambos (1969) and Gocke (1975). However, histological and IF evidence of immune complex deposition in synovial tissues containing HBsAg is lacking, and no convincing evidence of involvement of HBsAg in the pathogenesis of arthralgia or arthritis has been presented in the litera-

ture studied. The possibility of other hepatitis B associated antigens (e.g. hepatitis B core antigen: HBcAg) being responsible for the formation of pathogenic complexes should be considered. The appearance of anti HBc antibodies (anti HBc) at the beginning of clinical signs of acute hepatitis (Hoofnagle et al., 1973) coincides well with the expected sequence of serological events, if HBcAg-anti HBc immune complexes are responsible for skin and joint manifestations in the prodromal phase of acute hepatitis.

In the material studied, 2 cases of PAN were HBsAg seropositive, although in one of these cases HBsAg could first be demonstrated in the serum 6 weeks after the time that renal and skin biopsy specimens confirmed the vasculitis. HBsAg could not be demonstrated in renal glomeruli and vessel walls in the biopsy specimens in this case, using the IF-technique. Although in the second case of PAN it was also impossible to demonstrate HBsAg in the walls of the affected vessels, in this case no frozen tissue specimens were available for IF-studies. However, HBsAg could be demonstrated by IF in "ground glass" cells in a formalin fixed liver biopsy specimen from this patient, taken at about the same time as the skin, muscle and fascie biopsy specimens.

In 8 out of 19 cases of PAN, studied by light microscopical and IF techniques, HBsAg has been demonstrated in the vessel walls in association with Ig and C (Gocke et al., 1971; Nowoslawski et al., 1972; Heathcote et al., 1972; Gerber et al., 1972; Baker et al., 1972) and data from these studies indicate that no significant relation to any of the histological types of hepatitis can be recognized. Although the incidence of PAN in hepatitis B is very low, 30-40% of patients with PAN are reported to be HBsAg seropositive (Gocke, 1975).

Glomerulonephritis was not present in the 107 hepatitis B cases in our study. Neither could HBsAg be demonstrated in 93 cases of immune complex glomerulonephritis.

Therefore, the incidence of glomerulonephritis, as of PAN, in hepatitis B should be considered to be very low. In contrast to PAN however, the incidence of HBsAg in cases of immune complex glomerulonephritis is also very low. Two more differences of glomerulonephritis and PAN in their relation to hepatitis B are that 1. in all cases of immune complex glomerulonephritis reported in association with hepatitis B, glomerular HBsAg-deposits have been demonstrated by IF and/or electron microscopical examination (Combes et al.,1971; Nowoslawski et al., 1972; Myers et al., 1973; Knieser et al., 1974; Kohler et al., 1974; Brzosko et al., 1974; McIntosh et al., 1976) and 2. most (i.e. 8 out of 10) cases of glomerulonephritis, reported in association with histologically typed hepatitis, occurred in cases of chronic active hepatitis (CAH).

The results of the PMN phagocytosis test, applied to 35 cases of histologically typed hepatitis B, indicated that immune complexes were present in the serum from 5 out of 16 cases of CAH, and involvement of HBsAg in immune complex formation could be demonstrated in one of these cases. Only in two cases PMN inclusions were demonstrated unassociated with CAH, and both concerned cases of PAN with active vasculitis.

Since IF studies of liver tissue have demonstrated the presence of HBsAg along the cellular membrane of hepatocytes ("diffuse pattern" according to Roos et al., 1976) in CAH, it is conceivable that this membrane bound foreign antigen induces the host defence mechanism to a chronic breakdown of liver cells, ultimately resulting in fibrosis or cirrhosis. In addition, shedding of cell membrane bound antigen-antibody complexes may occur, and this is probably reflected by phagocytosis by PMN. When elimination of the "shedded" immune complexes is insufficient, tissue deposition in vessel walls or glomeruli with subsequent tissue damage may result.

The results of the clinicopathological studies reported in chapter V have demonstrated that the PMN phagocytosis test: 1. is able to detect immune complexes in sera from patients which either have manifest systemic disease (i.e. PAN) or are at risk to develop systemic disease (i.e. CAH), and 2. is able to detect the antigen involved in immune complex formation. Since the test can be performed in any laboratory equipped for IF studies, the technique seems to meet all three requirements postulated at the beginning of chapter VI. Comparative studies, including other immune complex detection techniques and further clinicopathological studies should be performed for evaluation of the results reported.

SUMMARY

In this thesis some experimental and clinicopathological studies are reported, which were designed with the purpose to develop a technique which enables to monitor diseases mediated by circulating immune complexes.

In chapter I the place of immune complex mediated diseases among other diseases, caused by immunologic mechanisms resulting in tissue damage, is outlined. In addition, the principles of the techniques, designed for measurement of antigen-antibody interaction are summarized, as well as the fate of circulating immune complexes in vivo.

In chapter II, technical details of an immunofluorescence procedure for PMN are discussed, since data from the literature indicated the occurrence of Ig and C inclusions in PMN, in the course of several diseases, presumably representing phagocytosis of immune complexes.

In chapter III comparative studies concerning the influence of several anticoagulants used for PMN isolation on the observations obtained by the IF technique are reported. In addition, in this chapter the influence of the antigen-antibody ratio on the in vitro phagocytosis of artificially prepared HBsAg-anti HBs immune complexes is demonstrated.

Since extra hepatic manifestations of hepatitis B are attributed to circulating immune complexes, the literature concerning systemic disease and immune complex detection techniques used in relation to HBsAg is reviewed in chapter IV.

In chapter V the incidence of signs and symptoms of systemic disease, possibly related to immune complex deposition in a series of patients with HBsAg associated hepatitis is reported. In addition, the occurrence of HBsAg in a series of cases of immune complex glomerulonephritis was screened, and the PMN phagocytosis test for the

detection of circulating immune complexes was applied to 35 patients with HBsAg-associated hepatitis. The results of these studies indicate that the PMN phagocytosis test:
1. is able to detect immune complexes in sera from patients who either have manifest systemic disease or are at risk to develop systemic disease, and 2. is able to detect the antigen involved in immune complex formation.

In chapter VI 3 major requirements are postulated for an immune complex detection technique in order to be of clinical interest. These requirements are:

1. The results of the test should correlate with clinical signs and symptoms of immune complex mediated disease, i.e. the test should discriminate potentially pathogenic from "harmless" complexes.
2. The test should include the possibility to detect the antigen involved in immune complex formation.
3. The laboratory outfit needed should be relatively simple, and only commercially available reagents should be necessary.

It is discussed that the results of the studies, reported in this thesis, indicate that the PMN phagocytosis test seems to meet all these requirements.

SAMENVATTING

De in dit proefschrift beschreven experimenten en het klinisch-pathologisch onderzoek werden opgezet met als doelstelling een techniek voor het aantonen van circulerende immuuncomplexen te ontwikkelen en te toetsen.

In hoofdstuk I worden de ziekten, veroorzaakt door circulerende immuuncomplexen geplaatst in het kader van die ziekten, welke veroorzaakt worden door weefselbeschadiging ten gevolge van een immunologische reactie. Tevens worden hier de principes besproken van de technieken die ontworpen zijn voor het meten van antigeen-antilichaam interacties. Tenslotte wordt het "lot" van immuuncomplexen in de circulatie aangegeven.

In hoofdstuk II wordt een immunofluorescentie procedure beschreven voor uit patientenbloed geisoleerde granulocyten. Deze techniek werd ontwikkeld, omdat uit literatuurgegevens blijkt dat in de loop van verscheidene ziektebeelden insluitsels in het cytoplasma van granulocyten kunnen voorkomen, die immunoglobulines en complement bevatten. Deze insluitsels vertegenwoordigen vermoedelijk gephagocyteerde immuuncomplexen.

In hoofdstuk III worden de resultaten besproken van enige vergelijkende onderzoekingen naar de invloed van verschillende anticoagulantia, gebruikt voor de isolatie van granulocyten uit bloedmonsters. Bovendien wordt in dit hoofdstuk aangetoond dat de antigeen-antilichaam verhouding van kunstmatig bereide HBsAg-anti HBs immuuncomplexen van invloed is op de in vitro phagocytose van deze complexen door granulocyten.

Een aantal van de extrahepatische ziekteverschijnselen van hepatitis B wordt toegeschreven aan circulerende immuuncomplexen. In hoofdstuk IV wordt een overzicht gegeven van de literatuur die betrekking heeft op systeem afwijkingen en immuuncomplex detectie technieken in relatie met HBsAg.

In hoofdstuk V worden de resultaten besproken van drie onderzoeken: In de eerste plaatse is nagegaan wat de incidentie is van ziekteverschijnselen die kunnen worden toegeschreven aan circulerende immuuncomplexen, in een groep van patienten met hepatitis B met histologisch geklasseerde leverafwijkingen. In de tweede plaats is onderzocht of HBsAg kon worden aangetoond in het nierweefsel van een groep patienten met een glomerulonefritis op basis van circulerende immuuncomplexen. Ten derde worden de resultaten besproken van de granulocyten-phagocytosetest, uitgevoerd bij 35 patienten met histologisch geklasseerde hepatitis B. De resultaten van dit onderzoek tonen aan dat de granulocyten phagocytosetest: 1. in staat is immuuncomplexen aan te tonen in sera van patienten die hetzij manifeste systeem aandoeningen hebben, hetzij een verhoogde kans hebben op het ontwikkelen van dergelijke aandoeningen, en 2. het antigeen dat betrokken is bij de vorming van immuuncomplexen kan aantonen.

In hoofdstuk VI worden 3 desiderata geformuleerd, waaraan een immuuncomplex detectie techniek zou moeten voldoen, om klinisch van betekenis te kunnen zijn.
Deze vereisten zijn:

1. De resultaten van de test dienen te correleren met het klinisch ziektebeeld, d.w.z. de test moet in staat zijn potentieel pathogene van onschadelijke immuuncomplexen te onderscheiden.
2. De test moet in principe in staat zijn, ook het antigeen aan te tonen dat betrokken is bij de vorming van de circulerende immuuncomplexen.
3. Bij voorkeur dient de techniek geen kostbare en ingewikkelde apparatuur te vereisen, en moet deze uitgevoerd kunnen worden met in de handel verkrijgbare reagentia.

De resultaten van de onderzoekingen, waarvan in dit proefschrift verslag wordt gedaan, wijzen er op dat de granulocyten phagocytosetest tegemoet lijkt te komen aan elk van deze vereisten.

REFERENCES

Chapter I, IV and VI.

Agnello, V., Winchester, R.J. and Kunkel, H.G. (1970). Precipitin reactions of the C1q component of complement with aggregated gamma-globulin and immune complexes in gel diffusion. Immunology 19: 909-919.

Alarcon, G.S. and Townes, A.S. (1973). Arthritis in viral hepatitis Report of two cases and review of the literature. Hopkins Med.J. 132: 1-15.

Almeida, J.D. and Waterson, A.P. (1969). Immune complexes in hepatitis. Lancet 2: 983-986.

Alpert, E., Isselbacher, K.J. and Schur, P.H. (1971). The pathogenesis of arthritis associated with viral hepatitis. Complement component studies. N.Engl.J.Med. 285: 185-189.

Alpert, E., Schur, P.H., Isselbacher, K.J. (1972). Sequential changes of serum complement in HAA related arthritis. N.Engl.J.Med. 287: 103 (abstr.).

Baker, A.L., Kaplan, M.M., Benz, W.C., Sidel, J.S. and Wolfe, H.J. (1972). Polyarteritis associated with Australia-antigen positive hepatitis. Gastroenterology 62: 105-110.

Benacerraf, B., Sebastijen, M. and Cooper, N.S. (1959). The clearance of antigen-antibody complexes from the blood by the reticulo-endothelial system. J.Immunol. 82: 131.

Boggs, D.R. (1967). The kinetics of neutrophilic leucocytes in health and in disease. Semin. Haemat. 4, 359.

Brzosko, W.J., Krawczynski, K., Nazarewicz, T., Morzycka, M., and Nowoslawski, A. (1974). Glomerulonephritis associated with hepatitis B surface antigen immune complexes in children. Lancet 2: 477.

Cats, A., Lafeber, G.J.M. and Klein, F. (1975). Immunoglobulin phagocytosis by granulocytes from sera and synovial fluids in various rheumatoid and non-rheumatoid diseases. Ann.rheum. Dis. 34, 146.

Cochrane, C.G., Weigle, W.O. and Dixon, F.J. (1959). The role of polymorphonuclear leucocytes in the initiation and cessation of the Arthus vasculitis. J.Exp. Med. 110, 481.

Combes, B., Stastny, P., Shopey, J., Eigenbrodt, E.H., Barrera, A., Hull, A.R. and Carter, N.W. (1971). Glomerulonephritis with deposition of Australia antigen-antibody complexes in glomerular basement membrane. Lancet 2: 234.

Dixon, F.J., Vazquez, J.J., Weigle, W.V. and Cochrane, C.G. (1958). The pathogenesis of serumsickness. Arch.Pathol. 65: 18.

Duffy, J., Lidsky, M.D., Sharp, J.T., Davis, J.S., Person, D.A., Hollinger, F.B. and Min, K.W. (1976). Polyarthritis, polyarteritis and hepatitis B. Medicine 55: 19-37.

Eknoyan, G., Györkey, F., Dichoso, C., Martinez-Maldonado, M., Suki, W.N. and Györkey, P. (1972). Renal morphological and immunological changes associated with acute viral hepatitis. Kidney Internat. 1: 413_419.

Farr, R.S. (1958). A quantitative immunochemical measure of primary interaction between I BSA and antibody. J.Infect.Dis.103, 239.

Gell, P,G,H. and Coombs, R.R.A. (1968). Clinical aspects of immunology 2nd Ed., F.A. Davis Co., Philadelphia, p. 575.
Gerber, M.A., Brodin, A., Steinberg, D., Vernace, S., Yang, C.P. and Paronetto, F. (1972). Periarteritis nodosa, Australia antigen and lymphatic Leucaemia. N.Engl.J.Med. 286: 14-17.
Gocke, D.J. (1975). Extrahepatic manifestattions of viral hepatitis. Am.J.Med.Sci. 270, 49-52.
Gocke, D.J., Morgan, C., Lockshin, M., Hsu, K., Bombardieri, S., Christian, C.L. (1970). Association between polyarteritis and Australia antigen. Lancet 2: 1149-1153.
Gocke, D.J., Hsu, K., Morgan, C., Bombardieri, S., Lockshin, M., Christian, C.L. (1971). Vasculitis in association with Australia antigen. J.Exp.Med. 134: 330S-336S.
Heathcote, E.J.L., Dudley, F.J. and Sherlock, S. (1972). The association of polyarteritis and Australia antigen. Digestion, 6: 280-281 (abstr.).
Hoofnagle, J.H., Gerety, R.J. and Barker, L.F. (1973). Antibody to hepatitis B-virus core in man. Lancet 2: 869-873.
Knieser, M.R., Jenis, E.H., Lowenthal,D.T., Bancroft, W.H., Burns, W., Shalhoub, R. (1974). Pathogenesis of renal disease associated with viral hepatitis. Arch.Pathol. 97: 193-200.
Kohler, P.F. (1973). Clinical immune complex disease. Manifestations in systemic lupus erythematosus and hepatitis B virus infection. Medicine 52, 419-429.
Kohler, P.F., Cronin, R.E., Hammond, W.S., Olin, D., Carr, R.I. (1974). Chronic membranous glomerulonephritis caused by hepatitis B antigen-antibody immune complexes. Ann.Int.Med. 81: 448-451.
McAuliffe, V.J., Kachani, Z.F. and Gocke, D.J. (1973). Biological activity of hepatitis B antigen soluble immune complexes. Lancet 2 . 968.
McCarty, D.J. and Ormiste, V. (1973). Arthritis and HBsAg-positive hepatitis. Arch.Intern.Med. 132: 264-268.
McIntosh, R.M., Koss, M.N. and Gocke, D.J. (1976). The nature and incidence of cryoproteins in hepatitis B antigen (HBsAg) positive patients. Quart.J.Med. 45 (69) 23-38.
Millman, J., London, W.T., Sutnick, A.J., Blumberg, B.S. (1970). Australia Antigen-Antibody Complexes. Nature 226, 83-84.
Minden, P. and Farr, R.S. (1971). Antigen-antibody interactions and their measurement. In: Immunological Diseases. Ed: M.Samter 2nd edition. Little, Brown and Comp., Boston, p. 179.
Mistilis, S.P. and Blackburn, C.R.B. (1970). Active chronic hepatitis. Am.J.Med. 48: 484-495.
Mosley, J. and Galambos, J. (1969). Viral hepatitis. In: Diseases of the Liver. Edited by Leon Schiff, J.B. Lippincot Co., Philadelphia, Toronto, p. 448-449.
Myers, B.D., Griffel, B., Naveh, D., Jankielowitz, T., Klajman, A., (1973). Membrano-proliferative glomerulonephritis associated with persistent viral hepatitis. Am.J.Clin.Pathol. 60: 222-228.
Nowoslawski, A., Krawczynski, K., Brzosko, W.J. and Madalinski, K. (1972). Tissue localisation of Australia antigen immune complexes in acute and chronic hepatitis and liver cirrhosis. Am.J.Pathol. 68: 31-50.

Nydegger, U.E., Lambert, P.H., Gerber, H. and Miescher, P.A. (1974). Circulating immune complexes in the serum in systemic lupus erythematosus and in carriers of hepatitis B antigen. Quantitation by binding to radiolabeled Clq. J.Clin.Invest. 54: 297-309..

Onion, D.K., Crumpacker, C.S. and Gilliland, B.C. (1971). Arthritis of hepatitis associated with Australia antigen. Ann.Intern. Med. 75: 29-33.

Parker, R.L. and Schmid, F.R. (1962). Phagocytosis of particulate complexes of gammaglobulin and rheumatoid factor. J.Immunol. 88: 519.

Prince, A.M. and Trepo, C. (1971). Role of immune complexes involving SH antigen in pathogenesis of chronic active hepatitis and polyarteritis nodosa. Lancet 1: 1309-1312.

Randall, R.E., Abukurah, A.R., Tung, M.Y., Vaugh, E.R. Still. W.J.S. (1971). Renal manifestations of chronic hepatitis. J.Clin.Invest. 50: 75a. (abstr.).

Rawson, A.J., Abelson, A.M. and Hollander, J.L. (1965). Studies on the pathogenesis of rheumatoid joint inflammation. II. Intracytoplasmic particulate complexes in rheumatoid synovial fluids. Ann.Intern.Med. 62: 281.

Roos, C.M. Feltkamp-Vroom, Th.M. and Helder, A.W. (1976). The localization of hepatitis B antigen and immunoglobulin G in liver tissue; an immunofluorescence, light and electron microscopic study. J.Pathol. 118, 1.

Sergent, J.S., Lockshin, M.D., Christian, C.L. and Gocke, D.J. (1976). Vasculitis with hepatitis B antigenemia: Long-term observations in nine patients. Medicine 55: 1-18.

Shulman, N.R. and Barker, L.F. (1969). Virus-like antigen, antibody and antigen-antibody complexes in hepatitis measured by complement fixation. Science 165: 304-306.

Sobel, A.T., Bokisch, V.A., and Müller-Eberhard, H.J. (1976). Clq deviation test for the detection of immune complexes, aggregates of IgG, and bacterial products in human serum. J.Exp.Med. 142: 139.

Steffelaar, J.W., de Graaff-Reitsma, C.B. and Feltkamp-Vroom, Th.M. (1976). Immune complex detection by immunofluorescence on peripheral blood polymorphonuclear leucocytes. Clin.Exp. Immunol. 23: 272-278.

Steffelaar, J.W., ten Kate, F.J.W., Nap, M., Swaak, A.J.G., De Graaff-Reitsma, C.B., v.Elven, E.H. and Feltkamp-Vroom, Th.M. (1977). Immune complex detection by immunofluorescence on polymorphonuclear leucocytes. Clin.Exp.Immunol.: accepted for publication.

Szmuness, W., Hirsch, R.L., Prince, A.M., Levine, R.W., Harley, E.J. and Ikram, H. (1975). Hepatitis B surface antigen in blood donors: Further observations. J.Infect.Dis. 131, 111-118.

Theofilopoulos, A.N., Wilson, C.B. and Dixon, F.J. (1976). The Raji cell radio immune assay for detecting immune complexes in human sera. J.Clin.Invest. 57: 169-182.

Trepo. C., Thivolet, J. and Labert, R. (1972). Four cases of periarteritis nodosa associated with persistent Australia antigen. Digestion 5: 100-107.

Trepo, C.G., Zuckerman, A.J., Bird, R.C. and Prince, A.M. (1974). The role of circulating hepatitis B antigen/antibody immune complexes in the pathogenesis of vascular and hepatic manifestations in polyarteritis nodosa. J.Clin.Path. 27: 863-868.

Udenfriend, S. (1969), Fluorescence assay in Biology and Medicine. Volume 2, p. 388. Academic Press, New York and London.

Vaughan, J.H., Barnett, E.V., Sibel, M.V. and Jacox, R.F. (1968). Intracytoplasmic inclusions of immunoglobulins in rheumatoid arthritis and other diseases. Arthr.Rheum. 11: 125.

Wands, J.R., Mann, E., Alpert, E. and Isselbacher, K.J.(1975). The pathogenesis of arthritis associated with acute hepatitis B surface antigen-positive hepatitis. Complement activation and characterization of circulating immune complexes. J.Clin. Invest. 55: 930-936.

Wenzel, R.P. McCormick, D.P. Busch,H.J., Beam, W.E. (1972). Arthritis and viral hepatitis. A patient with transient serum hepatitis-associated antigen, skin nodules, rash and low serum complement. Arch.Intern.Med. 130: 770-771.

Winchester, R.J. Kunkel, H.G. and Agnello, V. (1971). Occurrence of gammaglobulin complexes in serum and joint fluid of rheumatoid arthritis patients: use of monoclonal rheumatoid factors as reagents for their demonstration. J.Exp.Med. 134: 286s-295s.

Woolf, J.L. Jones, D.M., Tapp, E. and Dymock, I.W. (1975). Electron microscopy of serum of healthy hepatitis B antigen carriers. J.Clin.Path. 28, 260-262.

CURRICULUM VITAE

Na het behalen van het diploma Gymnasium B aan het Stedelijk Gymnasium te Haarlem en één jaar scheikunde studie aan de Rijks Universiteit te Leiden, werd ik in 1962 ingeschreven aan de Medische Faculteit, eveneens van de Rijks Universiteit te Leiden, alwaar ik in 1967 het doctoraal examen behaalde. In de jaren 1965/1968 was ik student-assistent resp. doctoraal-assistent op het laboratorium voor Pathologische Anatomie van het Academisch Ziekenhuis Leiden (Prof.Dr.Th.G.van Rijssel). In 1970 behaalde ik het artsexamen bij de Faculteit der Geneeskunde te Rotterdam en was aansluitend gedurende 2 jaar werkzaam als arts-assistent in de kindergeneeskunde in het Sophia Kinderziekenhuis te Rotterdam (Prof.Dr.H.K.A.Visser). Mijn opleiding tot patholoog-anatoom ontving ik in het Pathologisch Anatomisch Laboratorium van het Academisch Ziekenhuis Dijkzigt te Rotterdam (Prof.Dr.G.Wielenga) in de jaren 1972/1976, en sedert mijn inschrijving in het register van medische specialisten d.d. 1.4.1976 ben ik als zodanig in dat laboratorium werkzaam.

De basis voor het onderzoek, beschreven in dit proefschrift, werd gelegd tijdens een stage in de immunopathologie op de afdeling voor Pathologische Anatomie van het Centraal Laboratorium van de Bloedtransfusiedienst te Amsterdam (hoofd destijds: Mevr.Dr.Th.M.Feltkamp-Vroom). De nauwe samenwerking, welke voortduurde ook na de beeindiging van mijn stage op deze afdeling, was een voortdurende stimulans en onmisbaar voor de verkregen resultaten. Het klinisch-pathologisch onderzoek kwam mede tot stand dankzij de grote belangstelling voor de histologische aspecten van leverpathologie van de zijde van de afdeling Interne Geneeskunde II van het Academisch Ziekenhuis Dijkzigt.