almost as frequent an accompaniment of the former as of the latter. This role for liver cell damage is further supported by our findings that biochemical and clinical relapse in infective hepatitis seems to be accompanied by reappearance of S.M. antibody in the serum. Moreover, a positive antinuclear factor during the course of the illness may suggest persistent disease or relapse occurring. The patients will be followed further to obtain more evidence on this point.

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Australia Antigen and Autoantibodies in Chronic Hepatitis

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Summary: The sera of 110 patients with chronic hepatitis and adequate controls were examined for antibodies to smooth muscle (S.M.), mitochondria (M.), and for antinuclear factors by the immunofluorescence method, and for Australia (Au(1)) antigen by a modified micro-Ouchterlony immunodiffusion technique. Twelve out of 13 patients with primary biliary cirrhosis had M. antibodies, two had antinuclear factor, and none had Au(1) in their sera. In chronic aggressive hepatitis 23·5% of the sera contained antinuclear factor, 13% S.M. antibodies, 10·5% M. antibodies, and 22% Au(1) antigen. Of the 12 patients with chronic persistent hepatitis, one had antinuclear factor, one S.M. antibodies, and three Au(1) antigen.

The most striking finding was a mutual exclusion between Au(1) antigen and M. and S.M. antibodies. None of the 33 patients with one or the other form of chronic hepatitis and M. or S.M. antibodies had Au(1) antigen; 22 out of 77 (28%) patients without such antibodies were positive.

Introduction

Studies of the various autoantibodies in chronic hepatitis have already been extensively reported. These antibodies are usually detected by immunofluorescence. Antinuclear factors (Paronetto *et al.*, 1961), non-organ-specific cytoplasmic antibodies (M. antibodies) (Walker *et al.*, 1965), and antibodies against smooth muscle (S.M. antibodies) (Johnson *et al.*, 1965) are most frequently determined. Their presence is usually taken as an indicator of deranged immunity, and autoimmune processes are believed to play a part in the pathogenesis of this group of diseases.

The Australia antigen Au(1) is presumed to be a component of a virus which can cause active hepatitis (Blumberg *et al.*, 1969; *British Medical Journal*, 1970); it is identical wit' the SH antigen of Prince (1968b). At least two serological types of hepatitis virus exist, which differ in incubation period (Krugman *et al.*, 1967). Only serum of long-incubation-period hepatitis is Au(1)-positive (Giles *et al.*, 1969). Wright *et al.* (1969a) suggested that active chronic hepatitis is perpetuated in some cases by a hepatitis virus related to Au(1) antigen.

To date there has been no systematic study of the various autoantibodies in relation to the occurrence of Au(1) antigen in chronic hepatitis. The sera collected during a collaborative clinical study of chronic hepatitis in Switzerland were examined for antinuclear factor, M. and S.M. antibodies, and Au(1) antigen. In this report their incidence, especially in chronic aggressive hepatitis, is evaluated and the implications of the results on pathogenesis are discussed.

Patients and Methods

Sera

Sera were obtained from patients included in a study of chronic hepatitis (Swiss Society of Gastroenterology, Study Group of Chronic Hepatitis). Diagnosis of chronic hepatitis was made by histological examination together with the clinical, biochemical, and other findings. Chronic aggressive hepatitis and chronic persistent hepatitis are defined as described by DeGroote *et al.* (1968) and primary biliary cirrhosis (=chronic non-suppurative destructive cholangitis) as described by Rubin *et al.* (1965). Some of the patients had developed cirrhosis and some were receiving steroids, azathioprine, or both when serum samples were taken. Where multiple samples at different time intervals were available, only the first one was included in this study. Clinical data and changes in titres during the course of the disease will be reported elsewhere. The sex and age distribution of the patients studied is shown in Table I. In the group consisting of "various liver diseases" are included alcoholic cirrhosis (5), extrahepatic biliary obstruction (10), drug-induced cholestasis (5), reactive hepatitis (5), acute hepatitis (20), acute hepatitis, reconvalescence period (13). Normal sera were obtained from blood donors. The other control sera were sent to this laboratory for determination of antinuclear factor from patients of this hospital with various diseases and clinical suspicion of immune diseases. Some of the sera were kept at −20° C. for up to two years.

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Fluorescence Technique

The double-layer technique was used throughout this study. Unfixed 4μ thick cryostat sections of rat kidney and stomach were used. Blocks were kept up to one month at −70° C. The sections were covered with dilutions of patient sera for 30 minutes at room temperature, washed three times in phosphate buffered saline (*p*H 7·2) for 10 minutes, and then covered with fluorescein-conjugated antihuman immunoglobulin serum for 30 minutes and washed as before. Coded sections were examined with darkground illumination on a Reichert Diapan microscope fitted with a halogen lamp and EXIFL and CUTFL filters from Shandon.

The antihuman immunoglobulin serum was prepared by immunizing rabbits with human Cohn fraction II (Hyland); this antiglobulin reacted on immunoelectrophoresis with human IgG, IgA, and IgM and faintly with some minor β-globulin components. The globulin fraction was conjugated with fluorescein isothiocyanate (Baltimore Biologicals) and evaluated after the methods of Holborow and Johnson (1967). It had a fluorescein/protein ratio of 1·7 and contained 1/5 unit of anti IgG/ml. in the dilution used. The same conjugate was used throughout the study, and its reactivity was checked against reference sera with each group of tests.

Antinuclear Factors

Antinuclear factors were determined on rat kidneys. Titres of $\geqslant$1/20 were considered positive. The incidence in controls was: various hospital patients (without systemic lupus erythematosus) 13% in females, 5% in males; systemic lupus erythematosus 100%; seropositive rheumatoid arthritis 25%.

Cytoplasmic M. Antibodies

M. antibodies were seen with both rat stomach and kidney, giving a granular staining of parietal and other mucosal cells and of kidney tubules. Titres were recorded on kidney sections and considered to be significant if $\geqslant$1/20. One woman having M. antibodies was discovered among normal blood donors. Her transaminase levels were normal, but liver biopsy gave the histological picture of chronic persistent hepatitis. Of 175 sera from various hospital patients two were positive—a male patient with tuberculous pleuritis and a woman with splenomegaly and anaemia.

Smooth Muscle Antibodies

S.M. antibodies were identified by their staining of the walls of renal vessels and gastric muscularis mucosae. Only titres $\geqslant$1/40 read on the stomach sections were regarded as positive. Again two of the various hospital patients were positive, none from the normal controls. Sera having M. antibodies gave usually a weak fine linear coloration between smooth muscle fibres; this was clearly different from the uniform coloration of muscle fibres as described by Johnson *et al.* (1965). Only the latter was accepted as indicating S.M. antibody.

Australia Antigen

Tests for Au(1) antigen and antibodies were done with a micro-Ouchterlony double-diffusion technique as modified by Prince (1968a). Serum containing antibody to Au(1) in a titre of 1/8 was obtained from a patient with cancer of the bile duct; he had received 2 units of blood four weeks previously at laparotomy. When this antiserum was tested against Au(1) antigen it showed a reaction of identity with reference antisera provided by Dr. A. M. Prince, New York, and Professor J.-P. Soulier, Paris. The same occurred when our own Au(1) antigen was compared with antigens obtained from the same sources, using all three antibodies. Au(1) antigen pool and antiserum were kept in small aliquots at −70° C. and thawed before use. When testing unknown sera, antibody was placed in the centre well and reference antigen in two opposite wells; this allows detection of both antigen and antibodies in the sera to be tested. Slides were kept in a moist chamber at room temperature for seven days. About 90% of the lines could be clearly seen after 24 hours. In the group of various control patients 1·8% were positive for Au(1) antigen; 190 blood donors examined were negative.

Results

The incidence of antinuclear factor and S.M. and M. antibodies is given in Table I. M. antibodies occurred only in primary biliary cirrhosis and chronic aggressive hepatitis. S.M. antibodies were found in chronic aggressive hepatitis, in one case of chronic persistent hepatitis without special clinical features and in the serum of a patient in the reconvalescence period of acute hepatitis. All three antibodies occurred mainly in women.

The frequency of Au(1) antigen is recorded in Table II. In acute hepatitis the positive tests were distributed equally in both sexes. Au(1) antigen disappeared when the hepatitis subsided. In chronic aggressive hepatitis Au(1) antigen was found in 16 males and three females, with a total incidence of 22%. This presented interesting features, as can be seen in Table III; only cases without M. or S.M. antibodies were positive. Two women with antinuclear factor were the only patients with autoantibodies and Au(1) antigen at the same time. This was confirmed when all groups of chronic hepatitis were taken together. None of the 33 patients with M. or S.M. antibodies had Au(1) antigen, while 22 out of 77 (28%)

TABLE I.—*Antinuclear Factor and S.M. and M. Antibodies in Liver Diseases*

Disease Group*		No.	Mean Age (Range)	Antinuclear Factor Positive	S.M. Positive	M. Positive
Primary biliary cirrhosis	Females	12	55·5 (40–60)	2 (15%)	0	11 (92%)
	Males	1	51	0	0	1
	Total	13	55 (40–69)	2 (15%)	0	12 (92%)
Chronic aggressive hepatitis	Females	44	56·5 (31–74)	16 (36%)	11 (25%)	9 (20·5%)
	Males	41	50 (22–69)	4 (8%)	0	0
	Total	85	53·5 (22–74)	20 (23·5%)	11 (13%)	9 (10·5%)
Chronic persistent hepatitis	Females	4	55 (43–69)	1	0	0
	Males	8	41 (27–72)	0	1	0
	Total	12	45·4 (27–72)	1 (8%)	1 (8%)	0
Various liver diseases	Females	31	47 (18–77)	3 (10%)	1 (3%)	0
	Males	27	51 (26–79)	1 (4%)	0	0
	Total	58	49 (18–79)	4 (7%)	1 (2%)	0

* See section on methods for definition

TABLE II.—*Au(1) Antigen and Antibody in Liver Disease*

	No.	Au(1) Antigen Positive		Au Antibody Positive
		No.	%	
Acute hepatitis	20	13	65	0
Primary biliary cirrhosis	13	0	0	0
Chronic aggressive hepatitis	85	19	22	1
Chronic persistent hepatitis	12	3	25	0
Other liver diseases	38	1	3	1

TABLE III.—*Au(1) Antigen in Chronic Aggressive Hepatitis*

	Total No.	Au(1) Positive	
		No.	%
All cases	85	19	22
Without S.M. and M. antibodies	65	19	29
Without antinuclear factor and S.M. and M. antibodies	54	17	31

patients without such antibodies were positive. Repeated determination with intervals of five months up to more than two years on two or more subsequent serum specimens showed identical results in 24 out of 25 patients.

Discussion

In primary biliary cirrhosis the incidence of M. antibodies in this study compares well with that reported by Doniach *et al.* (1966). The lower frequency of S.M. antibodies in our series probably reflects differences in technique of detection. In the group with chronic aggressive hepatitis comparison is more difficult. In the present study this disease has been classified histologically after the criteria of DeGroote *et al.* (1968). Most of the other series refer to active chronic hepatitis; this group has the same histological picture, but is primarily a clinical entity with features of generalized disease. Therefore the relatively lower incidence of M. and S.M. antibodies in this study reflects mainly the difference in populations of patients. Progression of hepatitis to cirrhosis in our patients occurs at about equal frequency in the groups with and without autoantibodies.

The incidence of 22% of Au(1) antigen in patients with chronic aggressive hepatitis is comparable with the studies done in the United States (Wright *et al.*, 1969a) and Austria (Krassnitzky *et al.* 1970), but in England (Fox *et al.*, 1969) and Australia (Mathews and Mackay, 1970) a much lower incidence is reported. Epidemiological features play a predominant part in the frequency of Au(1) antigen. This study adds information for Au(1) antigen in an additional country. More interesting, however, is the trend to mutual exclusion between M. and S.M. antibodies on the one hand and Au(1) antigen on the other. In none of the previous studies were both these autoantibodies and Au(1) antigen looked for. In the report of Mathews and Mackay (1970) on the incidence of Au(1) antigen in Australia, a woman with "lupoid hepatitis," M. antibodies, and Au(1) in the serum is mentioned. Another patient with chronic active hepatitis and Au(1) antigen had no M. or S.M. antibodies. A recent abstract reports the same findings for S.M. antibodies; none of the patients with high titre of these antibodies had Au(1) antigen (Wright *et al.*, 1969b).

Au(1) antigen has been found by immunofluorescence in liver biopsies of several patients, including cases with acute hepatitis and two cases of chronic active hepatitis (Coyne *et al.*, 1970). All those who had Au(1) in the serum were positive on biopsy. This renders the presence of Au(1) antigen in the serum more significant. In 3 of the 26 positive biopsies, however, the liver showed no inflammatory changes. This is consistent with reports on healthy carriers of Au(1) antigen in the serum (Blumberg *et al.*, 1969). The question arises whether prolonged infection with an agent like Au(1) can account for the chronic inflammation found in the liver. Viral infection can lead to chronic inflammation which may depend on the immune response against the causative agent as seen in lymphocytic choriomeningitis in the mouse (Hotchin, 1962) and in viral hepatitis infection in the dog (Preisig *et al.*, 1966) The role of viral agents in the initiation and maintenance of chronic hepatitis remains unclear. A prolonged or relapsing viral infection might cause the progressive histological lesions seen in chronic aggressive hepatitis (Doniach *et al.*, 1970), perhaps with an inadequate immune reaction (Vischer, 1969). This may, in relation to Au(1) antigen, account for cases without M. and S.M. antibodies but with persisting Au(1) antigen as reported in this study.

Probably other viral agents may be related to chronic hepatitis. Acute hepatitis can be caused by at least two serologically different viruses (Krugman *et al.*, 1967). In one case of chronic aggressive hepatitis virus-like structures similar to members of the corona virus group were identified in the serum (Zuckerman *et al.*, 1970). The initiation of disease in the group with M. and S.M. antibodies may also be due to virus, but autoimmune mechanisms may play a major part in a subsequent self-perpetuating process as discussed by Doniach and Walker (1969). A liver-specific antigen has been described recently. Prolonged immunization in rabbits leads to a liver inflammation closely resembling chronic aggressive hepatitis with typical "piecemeal" necrosis (Kössling and Meyer zum Büschenfelde, 1968). Cells producing antibodies to this antigen have been found in biopsies of cases with chronic aggressive hepatitis (Meyer zum Büschenfelde *et al.*, 1969).

From the findings of others and the data presented here it seems possible that different pathways may lead to similar inflammatory liver diseases: persistent viral infection, perhaps associated with inadequate immune response on the one hand, and autoimmune processes induced by unknown factors leading to a self-perpetuating inflammatory reaction on the other.

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Enzyme Activity, Acidic Nuclear Proteins, and Prognosis in Human Breast Cancer

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Summary: The activities of the enzymes lactate dehydrogenase, 6-phosphogluconate dehydrogenase, and phosphohexose isomerase in primary human breast cancer biopsies are shown to be related to the time between mastectomy and recurrence of the cancer. These enzymes have higher activity in malignant breast tissues generally than in non-malignant breast tissues. In tumours from patients with long free periods these differences are not apparent.

Evidence is presented which suggests that two different types of breast cancer can be distinguished according to the relative amounts of phosphohexose isomerase and acidic nuclear proteins. It is suggested that this difference may be related to hormone responsiveness.

Introduction

We have previously described some biochemical differences between human mammary tumours and non-malignant mammary tissue (Smith, King, Meggitt, and Allen, 1966). The activities of the enzymes β-glucuronidase, phosphohexose isomerase, lactate dehydrogenase, isocitrate dehydrogenase, glucose-6-phosphate dehydrogenase (G-6-PD), and 6-phosphogluconate dehydrogenase (6-PGD) were measured. These enzymes were chosen because there is a considerable body of evidence that neoplastic tissues differ from normal tissues in carbohydrate metabolism (Aisenberg, 1961). There is also evidence that some of these enzymes may be correlated with important clinical characteristics in animal tumours, such as growth rate (Shonk, Morris, and Boxer, 1965) or hormone responsiveness (Rees and Huggins, 1960).

In our study the enzyme activities in the tumours were very variable, but the activities of several pairs of enzymes were correlated—for example, lactate dehydrogenase and 6-PGD; phosphohexose isomerase and G-6-PD. We concluded that, despite the high variability of the individual enzyme activities, the enzyme pattern—that is the relative activities of the enzymes to each other—had a certain constancy, and suggested that quantitative differences in enzyme activity patterns might be of some prognostic value. The acidic nuclear-protein content of the tumours was also measured, since there was evidence that the amount of these proteins was correlated with growth rate. This work has been extended, and the present paper is an attempt to assess the clinical significance of the results in the light of postoperative "follow-up" information.

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Materials and Methods

Tissue samples were obtained within 10 to 15 minutes of excision. All the malignant tumours were primary breast carcinomata. Non-malignant tissue was obtained from uninvolved areas of breast containing carcinoma or from patients with cystic glandular hyperplasia. The clinical diagnosis was confirmed by histological examination of the samples. Tissues were frozen on solid CO_2 and stored at −20°C. until used. The variables measured were not significantly altered after as long as six months' storage under these conditions. We have no evidence for the effect of the initial freezing on human tissues, but in rat liver and in mouse tumours the variables were unaffected by this treatment and remained stable thereafter for long periods at −20°C. (Shonk and Boxer, 1964, and personal observations). Analyses were generally performed within one month of excision.

Assay Methods.—These have been described in detail (Smith *et al.*, 1966). The assays were performed in three stages, each requiring a separate homogenization.

D.N.A., R.N.A., Total Protein, Phosphohexose Isomerase, and β-glucuronidase.—Slices (about 200 mg.) were cut from the frozen tissue and homogenized with a Silverson homogenizer (Silverson Machines Ltd., London) in 1·8 ml. of a solution containing KCl (0·15 M), $NaHCO_3$ (0·003 M) and edetic acid (0·006 M), *p*H 6·7. Portions of this homogenate were used to estimate D.N.A. (Burton, 1956) and total protein (Lowry, Rosebrough, Farr, and Randall, 1951). Phosphohexose isomerase activity was determined by measuring the amount of fructose-6-phosphate formed from glucose (Bodansky, 1954) after 10 minutes' incubation of enzyme and substrate at 37°C. in tris buffer (0·025 M *p*H 7·4). β-glucuronidase was measured, phenolphthalein β-glucuronide being used as substrate. The assay conditions were taken from the Sigma Chemical Co. (St. Louis, U.S.A.) Bulletin 105 (1951). Triton X-100 was added to the incubation mixture at a final concentration of 0·002% (v/v) to release any bound enzyme. Assay conditions for both these enzymes were chosen to give linear reaction rates over the period of incubation.

Dehydrogenases (Lactate Dehydrogenase, Isocitrate Dehydrogenase, G-6-PD, 6-PGD).—A second homogenate was prepared, as above, and centrifuged at 2,000 *g* for 30 minutes at 40°C. The supernatant was kept on ice until used. The enzyme activities were measured by recording the rates of change in absorbance at 340 mμ due to the oxidation or reduction of the appropriate nicotinamide adenine dinucleotide with a Unicam SP 700 recording spectrophotometer. The conditions were chosen to give maximal rates at *p*H 7·4. In all cases the measurements are based on zero-order activity curves. Details of the reaction mixtures used were given in a previous publication (Smith *et al.*, 1966).

Acidic Nuclear Proteins.—Frozen tissue (100-200 mg.) was homogenized in 3 ml. of ice-cold 0·25 M sucrose : 3mM $CaCl_2$