STUDIES OF AUTO-IMMUNITY AND CYTOTOXICITY

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Being a thesis submitted in application
for admission to the degree of Doctor of Medicine
of the University of Adelaide

The work embodied in the Thesis "Studies of Autoimmunity and Cytotoxicity" was carried out at the University of Adelaide, the Middlesex Hospital Medical School, and the University of Michigan.

The composition is my own.

The work described in Part I of the Thesis was carried out in collaboration with Dr. Earle Hackett, of the Institute of Medical and Veterinary Science, Adelaide. The clinical collection of cases, diagnosis and classification was done by myself, and the laboratory investigations carried out on the sera of the patients were carried out in the laboratories of Dr. Hackett.

The sera of patients used in the second part of the Thesis were collected by Dr. D. Doniach, of the Institute of Clinical Research, Middlesex Hospital Medical School.

The clinical data were collected by Dr. Doniach, and the diagnosis and classification of the patients was reviewed by myself together with Dr. Doniach.

All microphotographs of the Plates were taken by myself. The laboratory investigations carried out in Part II of the Thesis were my own work, with the exceptions to be detailed.

The histological diagnoses in Table 4 were made by myself.

The tissue cultures and fluorescent antibody studies were carried out by myself, with technical assistance from Dr. I.L. Solomon during the last 5 months of the 16 months of the study, while Dr. Solomon was on leave from the Department of Pediatrics, Children's Hospital, Columbus, Ohio.

Serum protein column chromatography, zone electrophoresis, sucrose density gradient ultracentrifugation, and immunoelectrophoresis were carried out by myself.

Routine complement fraction reactions and tanned-cell haemagglutination reactions were carried out by Mr. K.G. Couchman, F.I.M.L.T.

The estimations of desoxyribosenucleic acid set out in Table 36 were performed by Mr. C. Shapland, A.I.M.L.T., in the laboratory of Dr. I.M. Reitt of the Courtauld Institute of Biochemistry, Middlesex Hospital Medical School.

The work described in the third part of the Thesis was entirely my own, with the help of my technician, Mr. A. Smiltens.

PART I CLINICAL STUDIES OF THE AUTO IMMUNE COMPLEMENT-FIXATION (AICF) TEST.

CONTENTS

INTRODUCTION

The concept that the antibody producing cells of the body may in some circumstances function to its detriment has been extraordinarily fruitful in stimulating research. That the cells of this system are involved in thyroid disease, some haemolytic anaemias and some other conditions suspected of having an auto-immune aetiology is proved beyond all doubt.

The cause or causes of such a pathogenetic mechanism, if it can be proved to exist, are unknown, as is the intimate nature of the involvement of auto-antibodies, or the cells bearing antibodies, in disease processes.

Nevertheless, whether autoimmunity be a cause or an accompaniment of certain human diseases, the vast research effort which is being made in many countries at present towards an understanding of the interrelationships between reticuloendothelial system and the rest of the body can not fail to be fruitful. The study of immunopathology has linked research in many fields--haemolytic diseases, the allergic diseases, cancer, organ transplantation, etc.

The diseases in which autoimmunity is suspected to be the pathogenetic mechanism are numerous, and the relevant literature is exceedingly voluminous. In no human disease, with the possible exception of some haemolytic anaemias (Brit. Med. J., 1959), is the concept proven. Even in the haemolytic anaemias the steps leading from the combination

of antibody with erythrocyte antigens to haemolysis are not understood (Dacie, 1959). Most of the antibodies found in the haemolytic states do not cause haemolysis directly, but presumably shorten the life of erythrocytes by making them liable to early phagocytosis.

The primary aim of this study of auto-immunity is to provide and assess evidence as to whether or not the cells of the human lymphoreticular system, or the products of those cells, may cause disease.

The first work provided data for comparison of clinical status of patients with the results of serological tests.

Studies of thyroid disease led to a review of published work relating to thyroiditis, because more accurate diagnosis and classification of thyroid disease is necessary for the interpretation of antibody tests.

Subsequent projects led to considerations of the way antibodies may affect living cells. Finally studies leading to the development of a method of detecting cytotoxic effects are presented.

AUTO-IMMUNITY: DEFINITIONS AND TERMS.

The term "auto-immunity" is commonly used loosely. The use of the term in such a way that its meaning is ambiguous confuses the aims of research.

The word is an unfortunate choice in several respects, but its use has become accepted. "Immunity" had a legal meaning of freedom from taxes (Lancet, 1959), then was adopted to mean freedom from disease (often with the implication that such freedom was due to circulating antibodies), and has been further used to signify a process whereby antibody production is induced ("immunization"), not only to pathogenic organisms and their products but also to other substances, even those derived from the organism's own tissues. "Auto-allergy" (Gear, 1959) and "auto-clasia" (Mackay, Taft and Cowling, 1959), are alternative proposals which may be preferable but are not commonly used.

The term can have either of two implications. "Auto-immunity" may merely signify the involvement of the reticulo-endothelial system in disease, as evidenced by the formation of antibodies which combine with the components of the patient's own tissues. It is used in this sense in this thesis.

Alternatively "auto-immunity" may be used to define a process whereby cells of the antibody-producing system, as a result of deranged activity, cause disease in their own organism. The term "auto-immune pathogenesis" will be used when expressing this possibility in order to keep the distinction between this unproven concept and the

phenomenon of auto-immunity.

"Auto-antibody" refers to gamma-globulin which combines with the patient's own tissues in various tests adapted from older bacteriological techniques.

The definitions of "antigen" and "antibody" are controversial, and unsatisfactory without reference to each other. The terms will be used with their usual significance in immunology. "Immunization" now connotes the injection of any substance ("antigen") with the intention of evoking antibody response. Immunity and antibody formation should not be considered synonymous. Unwieldy words such as "antibodization" (Fan, 1959) or "antigenation" (Gooch, 1959) seem to be the only alternative.

"Reticulo-endothelial system" (R.E.S.) refers to the populations of cells concerned with the antibody response. It includes the lymphocytes. "Lympho-reticular system" is used interchangeably. The "immunologically competent cell," as defined by Burnet (1961) is one that differs from other morphologically and physiologically similar cells by specific reactivity with a particular antigen or antigenic determinant. The nature of the reaction induced is immaterial, e.g. production of erythema, or antibody. Since the passive transfer of auto-antibodies seems to be a harmless procedure, at least in the case of autoantibodies present in thyroiditis and systemic lupus erythematosus (as will be discussed), it is likely that disease resulting from auto-immunity is mediated by immunologically competent cells.

EVIDENCE OF AN AUTO-IMMUNE PATHOGENESIS OF CERTAIN HUMAN DISEASES

Evidence of the auto-immune pathogenesis of human diseases is mostly of an indirect nature.

Morphological evidence is presented by the finding of lymphocytes and plasma cells in a lesion, although there is no specific pattern by which lesions in auto-immune conditions may be distinguished from chronic inflammatory diseases of known aetiology.

Secondly, evidence is obtained by the detection of auto-antibodies by means of classical serological techniques or more recently devised methods. These techniques merely prove the presence of circulating globulins capable of combining with a tissue component. They do not take account of the availability of that component for combination in the body, nor has it been shown that such combination is detrimental to the tissue in vivo. A good example is to be found in thyroid disease, where serological studies have proved a significant association between the existence of disease and the occurrence of thyroid antibodies. A significant proportion of persons who have no clinical evidence of thyroid disease, however, have antibodies in their serum. It must be stated categorically that the demonstration of circulating auto-antibodies does not prove an auto-immune pathogenesis of any disease.

A third body of evidence is provided by the immunological production of lesions in experimental animals analogous to those of the human disease. Such experiments prove that disease can be produced through derangement of the antibody-producing system. It is not clear to what extent the results

of these experiments can be extrapolated to the analogous human condition.

The use of adjuvant substances combined with antigen is usually necessary to cause disease, showing that something more than exposure to free antigen is required. The action of adjuvants is not understood.

The correspondence between the form of human and the animal lesions is not exact, although the histological features of experimentally induced thyroiditis in the rat are very similar to those of Hashimoto's disease (Jones and Roitt, 1961). However, the lesions regress completely when antigenic stimulation is withheld. Therefore, although immunological production of lesions in experimental animals provides further indirect evidence, it does not constitute proof of auto-immunity in the corresponding human disease.

Witebsky (1957) has stated four criteria which should be fulfilled in order to prove "the connection of pathological changes to auto-immunization":-

1. Demonstration either of circulating antibodies active at body temperature in the serum of patients suffering from the disease, or cell bound antibodies by indirect means.
2. The antigen against which the antibody is directed should be characterized or even isolated.
3. Antibodies should be produced against the same antigen in experimental animals.
4. Pathological changes should appear in the corresponding tissue of an actively sensitized animal that are similar or identical to those seen in the human disease.

It is suggested that a further condition should be added to Witebsky's criteria:-

5. Final proof of an auto-immune pathogenesis should be achieved by demonstrating that antibodies or immunologically competent cells cause the lesion in question, when these are allowed to come in contact with the cells of the tissue involved.

Ideally this should be accomplished in a living human, by transfer of auto-antibodies or immunologically competent cells. This must have been done many times by transfusion of blood; no transfer of auto-immune disease has been noticed. Transfer of immunologically competent cells, except in the case of identical twins, must have, at most, a transient effect, because of rejection of homografted cells. Therefore experiments with tissues in vitro are more likely to be of value because some ethical and practical problems are avoided.

Demonstration of the capacity of an auto-antibody to modify a defined metabolic process would help to fulfill this requirement. This suggestion may best be explained by citing an hypothetical example. Enhancement, inhibition or alteration of reaction A→B occurring in an experimental system, in vitro, in response to the addition of auto-antibody to the system, would constitute evidence of metabolic activity of auto-antibodies. Evidence of any function of auto-antibodies is almost entirely lacking.

THE AUTO-IMMUNE COMPLEMENT FIXATION REACTION

Gajdusek (1957, 1958), while trying to detect the antigen of infectious hepatitis virus, discovered that extracts of liver and other tissues fixed complement with serum from patients suffering from a number of different diseases. In particular the reaction occurred in systemic lupus erythematosus, hepatitis accompanied by the L.E. phenomenon ("lupoid hepatitis") and other cases of hepatitis and macroglobulinaemia. He referred to numerous reports, mainly in the German literature, of earlier studies of antibodies detected by complement fixation and other techniques in rheumatic fever, glomerulonephritis, lupus, multiple sclerosis and liver disease.

Gajdusek found that sera would react with any number of a group of antigens prepared from liver, kidney, skeletal muscle, spleen, cardiac muscle, pancreas, thyroid and adrenal. In some cases there was a reaction with only one of these, but most commonly with several. Pancreas was a poor reactor, possibly because of the action of its enzymes on the substances reacting as antigens. Human embryo and rat tissues reacted similarly to extracts of adult human organs. Different batches of liver, kidney, thyroid and adrenal antigens prepared from tissues obtained from different patients yielded much the same reactions, but muscle, spleen and heart antigens were more variable and were less stable on storage.

Gajdusek reported several observations on the nature of the reagents. The heat sensitivity of the serum reagents

was similar to that of many classical complement fixing antibodies, being completely destroyed by exposure to 70° C for 30 minutes in one instance and at 65° for 30 minutes in another. The activity was eluted from the gamma globulin fraction of an electrophoretic strip in one case; the gamma-globulin precipitate of several sera contained the activity; it was found in the precipitated macroglobulin of the case of paraproteinaemia.

The antigens of liver and kidney were stable for months at -20° C and were not destroyed by repeated freezing and thawing. They were destroyed by heating to 60° C for 30 minutes. Adrenal and thyroid antigens were almost as stable under these conditions, but muscle and splenic antigens did not withstand cold storage or heat as well. Rabbits and guinea pigs immunized with human liver or kidney suspensions developed complement fixing antibodies to human muscle, adrenal and thyroid antigens, and to rat liver, kidney and spleen antigens. These facts were taken as evidence in favor of regarding the A.I.C.F. serum reagents as true circulating antibodies.

Liver, kidney and muscle antigens were submitted to two cycles of centrifugation at 35,000 g. for 45 minutes with resuspension of the first sediments in isotonic saline to the original volume. The second sediments were found to contain virtually all of the antigenic activity. However, serum from one case of lupoid hepatitis reacted to a comparable titre with the sediments, washed particles and the unsedimented soluble components.

Mackay and Gajdusek (1958) further elaborated the

clinical relationships of the phenomenon. Striking association (high titres and frequent occurrence) was found between the occurrence of auto-antibodies and the following diseases:-

Liver disease (acute viral hepatitis, active chronic hepatitis, lupoid hepatitis, chronic nutritional hepatitis, chronic biliary cirrhosis).

"Collagen" diseases. Particularly systemic lupus erythematosus.

Paraproteinaemia. Two of 5 cases of macroglobulinaemia.

However, there was no relationship to the clinical manifestations of organ involvement and serum reactivity with the corresponding antigen; for example in lupus erythematosus reaction with kidney antigen occurred in the absence of clinical, laboratory and biopsy evidence of renal disease.

No laboratory test (including the gamma globulin level) showed a close correlation with positive A.I.C.F. titres.

THE AUTO-IMMUNE COMPLEMENT FIXATION REACTION IN 1,014 PATIENTS

This study was intended to extend the observations of Mackay and Gajdusek (1958), and to establish further clinical correlations of the clinical findings with the results of serological tests in which complement fixation was carried out with the patient's serum using saline extracts of human liver, kidney, adrenal and lung as antigens.

The methods and results are detailed in the appended publication. The serological tests were carried out in the laboratories of Dr. Hackett. All of the patients admitted to the Medical Professorial Unit, forming the bulk of the series were under my personal care, and most of the patients in the diffuse "collagen disease" group, thyroid disease, para- and dysproteinaemia, and myeloproliferative disorders group were studied at the bedside.

Data on cases of systemic lupus erythematosus para- and dysproteinaemia, Sjogren's disease, hepatic cirrhosis and acute hepatocellular disease are tabulated and discussed separately.

THE A.I.C.F.T. IN SYSTEMIC LUPUS ERYTHEMATOSUS

10 of 13 cases (77%) of systemic lupus erythematosus reacted positively with one or more test antigens. This compares with 9 positive reactions out of 12 cases of Gajdusek (75%). Another of his cases reacted to a titre of 4 with kidney and adrenal and, inexplicably, to 256 with spleen.

Intensive study of the 13 cases revealed no correlation between the disease manifestations and the A.I.C.F. results (Table 1). Increased dosage of cortisone in one case (L.3) coincided with a fall in titres from 160 (tested with liver, kidney, adrenal and thyroid) to 80, which coincided with symptomatic improvement. When tested again 4 months later (during pregnancy) the titres were again 160, and the same titres were found 6 weeks after parturition.

The thyroglobulin-tanned cell haemagglutination test was performed on the sera from these 13 patients. High titres were found in 3 of these, and a low titre in another. One of the cases with a high titre had a goitre which was diagnosed as non-toxic nodular goitre. The AICFT was positive only with normal thyroid antigen, also to a high titre.

Another had a non-toxic nodular goitre which had recurred after partial thyroidectomy seven years previously. This patient had no thyroid antibodies.

Thyroid disease was not apparent clinically in the other 3 cases having thyroglobulin antibodies.

TABLE I. A.I.C.F. AND T.C.H. (thyroglobulin) REACTIONS IN 13 CASES OF SYSTEMIC LUPUS ERYTHEMATOSUS.

	SEX	AGE	LIVER	KIDNEY	ADRENAL	LUNG	THYROID	T.C.H. (THYRO-GLOBULIN)	PRINCIPAL CLINICAL FEATURES
L1.	F	56	10	10	10	10	10	-	Recurrent nodular goitre. Arthritis. Leg ulceration.
L2.	F	57	80	80	80	10	80	-	Thrombocytopenic purpura.
L3.	F	32	160	160	160	0	160	40	Arthritis. Anaemia. Leucopenia. Leg ulceration.
L4.	F	17	10	10	10	10	10	-	Rash. Pneumonitis. Leg ulceration.
L5.	F	48	80	80	80	80	80	10240	Arthritis.
L6.	F	44	-	-	-	-	-	-	Remission. Arthritis suppressed by cortisone
L7.	F	47	-	-	-	-	-	-	Arthritis.
L8.	F	43	80	80	80	80	80	-	Nephrotic syndrome.
L9.	F	46	80	10	80	10	80	-	Nephrotic syndrome.
L10.	M	48	-	-	-	10	-	-	Transient arthropathy. Hypertension.
L11.	M	34	-	-	-	-	-	-	Rash, acute illness.
L12.	F	50	10	10	-	10	10	10240	Rash, fever, following discoid L.E. for 20 years.
L13.	F	44	-	-	-	-	80	10240	Haemolytic anaemia, nodular goitre.

TABLE 2. A.I.C.F.T. IN SYSTEMIC LUPUS ERYTHEMATOSUS

Number tested	13
Positive with any antigen	10
" " liver	8
" " kidney	8
" " adrenal	7
" " lung	7/12
" " thyroid	9

TABLE 3. THYROGLOBULIN ANTIBODIES (T.C.H. test) IN S.L.E.

Number tested	13
Number positive	4
Number of positives with recognizable thyroid disease	1
Thyroid disease without T.C.H. antibodies	1

THE A.I.C.F.T. IN PARA- AND DYSPROTEINAEMIA SYNDROMES

Eleven cases were available for study. The diagnosis was firm in nine, probable in one (D4) and made by another physician in one (D10). There was histological diagnosis in seven.

Two proven cases of macroglobulinaemia (D1, D2) gave positive A.I.C.F.T., and in the other two positive cases (D3, D10) diagnosis was less precise. Case D3 had hepatosplenomegaly, normochromic normocytic anaemia and grossly abnormal serum proteins. The anaemia was normocytic, the haemoglobin value being 9.5 g/100ml. The white cell count was 9.700 per c.mm. and was not abnormal. Electrophoretic analysis showed a low albumin (3.53 g/100ml), and elevated gamma globulin (2.67 g/100ml) with a fairly distinct peak and no beta gamma veil, which is usually seen in hepatic cirrhosis. Bence-Jones protein was not detected and the Sia water test for macroglobulin was negative. Bone marrow biopsy showed a slight increase in numbers of plasma cells of normal appearance, but no disturbance of the marrow structure.

The two cases of macroglobulinaemia tested had positive reactions in the A.I.C.F.T., one case of lymphosarcoma studied was negative, and one of seven cases of multiple myeloma was positive.

Serum from the positive case of multiple myeloma was supplied by a physician from another hospital and no clinical data are available.

It therefore appears that the A.I.C.F. phenomenon is

likely to be positive in a significant proportion of cases of that type of neoplastic disease of the reticuloendothelial system which is characterized by the production of macroglobulins, but not in multiple myelomatosis. This may be due to the broadness of the spectrum of globulins produced. In multiple myeloma a relatively homogeneous protein is produced by the tumor cells but the amount of normal globulin produced is probably small. This explanation may also hold for the low incidence of positive A.I.C.F. tests leukaemia and the myeloproliferative disorders.

TABLE 4. A.I.C.F.T. IN MACROGLOBULINAEMIA, MULTIPLE MYELOMA AND ALLIED CONDITIONS

	SEX	AGE	LIVER	KIDNEY	ADRENAL	LUNG	THYROID	DIAGNOSIS
D1	F	49	-	80	-	80	-	Macroglobulinaemia. Proved by ultracentrifuge studies. Histology, round cell infiltration of R.E. system. Narrow protein band.
D2	M	70	-	80	-	-	80	Macroglobulinaemia, proven. Histology, round cell infiltration. Narrow protein ban
D3	F	64	80	10	80	80	10	Paraproteinaemia, type not proven. Increase of normal plasma cells in bone marrow. Fairly narrow protein band.
D4	F	57	-	-	-	-	-	Lymphosarcoma with narrow protein band. Sia test for macroglobulins positive.
D5	M	46	-	-	-	-	-	Multiple myeloma, proven. Histology, plasma cell infiltration. Multiple myeloma band, Bence-Jones proteinuria
D6	M	69	-	-	-	-	-	Multiple myeloma, proven. Histology, myeloma cell infiltration. Slow beta band, low gamma.
D7	M	53	-	-	-	-	-	Multiple myeloma, no histological proof. Multiple myeloma band, Bence-Jones proteinuria.
D8	M	59	-	-	-	-	-	Multiple myeloma, no histological proof. Radiographic evidence. Myeloma band.
D9	M	70	-	-	-	-	-	Multiple myeloma. No histological proof. Radiographic evidence. Fairly broad protein band gamma globulin 5.28g/100ml.
D10	M	62	80	80	80	80	80	Multiple myeloma. Clinical diagnosis. No data available
D11	M	74	-	-	-	-	-	Multiple myeloma, proved at autopsy. Died before investgations completed.

THE A.I.C.F.T. IN SJÖGREN'S DISEASE

Bloch et al (1960) reported a high incidence of A.I.C.F. in Sjögren's disease, the salivary and lachrymal lesions of which have many histological similarities to Hashimoto's disease, together with a high incidence of thyroid antibodies, rheumatoid factor, precipitating antibodies to salivary gland and other tissue extracts and elevated serum gamma globulin levels. This disease, like systemic lupus erythematosus, is undoubtedly accompanied by immunological derangements and the presence of A.I.C.F. antibodies may be taken as an indication of immunological activity in a broad sense, but much more needs to be known before their precise significance can be envisaged.

Three cases of Sjögren's disease were studied (Table 5). A.I.C.F. antibodies were present in two, and thyroglobulin antibodies in two. There was no clinically apparent thyroid disease in these patients.

The serological reactions in this disease are complex. Work so far has hardly proceeded beyond showing the complexity. New reactions have been reported by Anderson et al (1961); there are two and possibly three precipitating antibody-antigen systems in which sera react with extracts of various human and animal tissues, and leucocytes from lymphatic but not myeloid leukaemia.

The studies with fluorescent antibody staining by these authors were incomplete. They reported that they had already found two types of nuclear fluorescence--homogeneous and "speckled", and also diffuse cytoplasmic fluorescence with

some sera. Some sera also reacted in the complement fixation test, and complement fixation correlated with one type of precipitating antibody.

Anderson et al also suggested that Sjögren's disease is like systemic lupus erythematosus in that antibodies to a wide range of antigens occur. Another point of similarity is the common association of Sjögren's disease with Hashimoto's thyroiditis (see Bunim, 1961).

TABLE 5. A.I.C.F.T. IN SJOGREN'S DISEASE

CASE	SEX	AGE	LIVER	A.I.C.F. KIDNEY	A.I.C.F. ADRENAL	A.I.C.F. THYROID	TCH (thyro-globulin)	ASSOCIATED CONDITIONS
S1	F	56	10	-	-	10	-	Duodenal ulcer.
S2	F	37	80	80	80	80	10	Arthralgia. Histo-logical confirmation of disease (parotid tissue).
S3	F	65	-	-	-	-	320	Rheumatoid arthritis, Rose's test 1/512, hepatomegaly.

TABLE 6 AICFT IN HEPATIC CIRRHOSIS

PATIENT	SEX	AGE	DIAGNOSIS	AETIOLOGY	AICF				SIZE OF LIVER	COLLOIDAL GOLD	CEPHALIN COLEST.	$Zn SO_4$	ALB	GAMMA GLOB.	THYROID	TCH THYRO-GLOBULIN	
					LIVER	KIDNEY	ADRENAL	THYROID									
C1	F	58	Cirrhosis	I.H.	-	-	-	-	++++	4	2	16			-		
C2	F	46	"	A	-	-	-	-	+				3.1	1.86	-		Alcoholic. Peripheral neuritis.
C3	F	61	"	A	-	-	-	-	++++	4	3	18	2.70	1.7.	-		
C4	F	67	"	? A	-	-	-	-	++	1	3	15			-		
C5	F	67	"	?	-	-	-	-	-	0	2		N	N	-		Autopsy proof.
C6	F	74	"	?	80	80	80	-	-	3	2	15	2.5	1.59	-		
C7	M	43	"	A	-	10	40	-	++++	5	4	16.1	2.21	1.12	-		Biopsy proof.
C8	F	51	"	?	-	-	-	-	-	4	4	14	2.12	1.39	-		Biopsy proof.
C9	F	67	"	A	-	-	-	-	-	4	4	18	3.02	1.12	-		Autopsy proof.
C10	M	40	"	A	-	-	-	-	++++	2	3	20	3.60	1.72	-		Ascites and telangiectases.
C11	M	40	"	?	-	-	-	-	++++	0	0	0	4.34	1.16	-		Haematamesis. Chronic alcoholic.
C12	F	38	"	A	-	-	-	-	++	4	4	13	3.9		-		Alcoholic. Also pregnant.
C13	M	63	"	?	-	-	-	-	+	1	3	13	4.18	1.4.	-		Biopsy proof.

TABLE 6 - (continued)

PATIENT	SEX	AGE	DIAGNOSIS	AETIOLOGY	AICP				SIZE OF LIVER	COLLOIDAL GOLD	CEPHALIN COLEST.	$ZnSO_4$	ALB	GAMMA GLOB.	THYROID	TCH THYRO-GLOBULIN	
					LIVER	KIDNEY	ADRENAL	THYROID									
C14	M	62	Cirrhosis	?	-	-	-	-	++	4	4		3.22	1.37	-		Biopsy proof.
C15	M	51	"	A	-	-	-	-	++	4	3	8.5	3.54	0.95	-		Biopsy proof.
C16	M	56	"	?	-	-	-	-	+++	5	4	35	3.0	1.24	-		Biopsy proof.
C17	F	43	"	?	-	-	-	-	-	0	1	13	1.84	1.58	-		
C18	M	34	"	A	-	-	-	-	++	3	3	19	2.50	1.63	-	-	Biopsy proof.
C19	M	77	"	?	-	10	-	-	+++		2	9.5	3.11	1.14	-	-	X-ray proof of varices. Splenomegaly.
C20	M	65	"	A	-	-	-	-	+++	3	1				-	-	Alcoholic. Long standing cirrhosis.
C21	M	54	"	A	-	-	-	-	+++		1	"	2.15	-	-		Biopsy cirrhosis? Biliary alcoholism & biliary calculus.
C22	F	61	"	A	-	-	-	-	+++		1				-		Biopsy proof.
C23	F	57	"	?	-	-	-	-	++	5	4	22		1.82	-	160	X-ray evidence of varices & portal vein occlusion
C24	M	69	"		-	-	-	-	+++	4	3		3.05	1.60	-		Autopsy proof. Biliary cirrhosis.
C25	M	39	"	A	-	-	-	-	++	5	4	9.3	N	+	-	-	Biopsy proof.

TABLE 6 - (continued)

PATIENT	SEX	AGE	DIAGNOSIS	AETIOLOGY	AICF				SIZE OF LIVER	COLLOIDAL GOLD	CEPHALIN COLEST.	$ZnSO_4$	ALB.	GAMMA GLOB.	THYROID	TCH THYRO-GLOBULIN	
					LIVER	KIDNEY	ADRENAL	THYROID									
C26	M	36	Cirrhosis	A	10	10	10	-	++++		4		low	N	-	-	
C27	M	63	"	A	-	-	-	-	++		2	20	low	+	-	640	Biopsy. Also has pulmonary T.B.
C28	F	63	"	?A	10	10	10	10	+				low	+	-		Ascites.
C29	M	46	"	?	10	10	10	10	+++	3	3	14.5	low	+	-	-	
C30	F	54	"	?	10	10	10	10	+++	2			N	+	-	-	
C31	M	49	"	A	10	10	10	10	+++				low	N	-	-	Alcoholism. Haematemesis.
C32	M	71	"	?	80	80	80	80	-	3	4	19	low	+	-	-	Biopsy proof.
C33	F	38	"	?	10	10	10	10	+++		2	+	2.62	1.33	-		Biopsy.
C34	F	56	"	I.H.	10	10	10	10	++		2		2.68	1.10	NOD	1:240	
C35	F	60	"	?	-	10	-	-	++	5		24.5	1.81	3.98	-		Autopsy proof.
C36	F	42	"	I.H.	-	10	80	-	-	5	4	25	1.9	2.4	-		Ascites.
C37	F	45	"	?	-	-	-	-	+				N	N	-		Biopsy proof.
C38	M	71	"	?	-	-	-	-	++						-	-	Haematemesis. Varices visualized.
C39	F	23	"	I.H.	-	-	-	-	-						-		Satisfactory clinical evidence. Post-hepatitic cirrhosis.

TABLE 6 - (continued)

PATIENT	SEX	AGE	DIAGNOSIS	AETIOLOGY	AICF				SIZE OF LIVER	COLLOIDAL GOLD	CEPHALIN CHOLEST.	$ZnSO_4$	ALB	GAMMA GLOB.	THYROID	TCH THYRO-GLOBULIN	
					LIVER	KIDNEY	ADRENAL	THYROID									
C40	M	64	Cirrhosis	A	-	-	-	-	≠	2	4	15.2	2.05	1.62	-	-	
C41	M	52	"	A	-	-	-	-	-	0	2	16	N	≠	-	80	Autopsy proof.
C42	M	62	"	A	-	-	-	-	≠≠	3	4				-	-	Chronic alcoholism, jaundice, haematemesis.
C43	F	55	"	?	80	80	80	80	≠				low	≠	-	10240	Biopsy proof.
C44	M	61	"	?	-	-	-	-	-						-		Biopsy. Splenectomy for Banti's syndrome.

THE A.I.C.F.T. IN HEPATIC CIRRHOSIS

Data on 44 cases of cirrhosis of the liver are presented here. These formed the bulk of the series reported in Hackett, Beech and Forbes (1960a), which was enlarged by 16 further cases from various sources after the following data had been assembled.

Of the 44 cases, 22 were proven histologically, and a further 3 had radiological evidence of oesophageal varices. Cases not proven histologically were accepted only if the clinical evidence was unequivocal. All had at least one of the following major manifestations: hepatomegaly, haematemesis, ascites, jaundice, peripheral oedema, low serum albumin level combined with high gamma globulin levels, accompanied by confirmatory evidence such as a history of prolonged heavy alcoholic consumption, telangiectases, splenomegaly, macrocytic anaemia, peripheral neuritis, positive flocculation tests (cephalin cholesterol, colloidal gold) and an elevated zinc sulphate value.

These features were considered individually in order to find a correlation between a positive A.I.C.F. test and the clinical state of the patient. There was no apparent relationship between any of these or any combination, or to age, sex, aetiology (whether associated with alcoholism or not) duration of cirrhosis and rapidity of progression. Liver biopsy sections were studied for the presence of a qualitative or quantitative difference between positive and negative reactors. No feature or pattern common to those reacting positively was found. Furthermore, there was no

common pattern of reaction to the antigens tested.

Data are available on the gamma globulins in 35 cases. The fractions were determined quantitatively in the Biochemistry Division of the Institute of Medical and Veterinary Science, Adelaide, by elution of stained strips cut into appropriate sections. (Method published in Hackett, Beech and Roman, 1960). Quantitative electrophoretic estimations were done on 23, in which the figures are quoted, and the information on the other 12 was either in the form of a scanning curve or a figure of globulin concentration obtained by a salting out procedure. The result is recorded as normal or increased in these cases (N or ↗).

The upper limit of normal accepted by the laboratory is 1.0 g/100ml. This figure is based on the results of testing a series of normal persons. The upper limit of normal accepted by Owen (1958) is 1.60 g/100ml.

The gamma globulin levels were greater than 1.0 g/100ml in 22 of 23 cases in which quantitative electrophoretic determination was done. The levels were 1.60 g/100 ml or greater in 7 of 23 cases.

The albumin level was below 3.8 g/100ml in 22 of 24 cases where it was determined quantitatively and was low in a further 7 of 12 cases where a scan curve or data of the albumin precipitation estimation were available.

A positive A.I.C.F. result was not connected with either very low albumin or high gamma globulin figures, or a pronounced inversion of the albumin/gamma globulin ratio.

In 4 cases the cirrhosis was thought probably to have been the result of previous infectious hepatitis, and in 2 of these the A.I.C.F. phenomenon was positive, but with liver antigen in one case only.

TABLE 6 A.I.C.F.T. IN HEPATIC CIRRHOSIS

Number	Positive with any antigen	Positive with			
		Liver	Kidney	Adrenal	Thyroid
44	14 (32%)	10 (23%)	14 (32%)	12 (27%)	8 (19%)

As in the overall survey, and in several of the sub-groups reaction with kidney was more frequent than with liver. Claims have been made that renal changes are more frequent in cirrhotics than are found in the whole at autopsy (Patek, Seegal and Bevans; 1951, Spellberg; 1955), but there was no obvious clinical evidence of renal disease in this series either in those with auto-antibodies or those without.

The metabolic interrelationships between the thyroid and the liver (Zimmerman, 1958) and the current clinical suspicion of a significant association of Hashimoto's disease with cirrhosis (summarized by McConkey and Callaghan, 1960) prompted an enquiry into the thyroid status of the cirrhotics in this series. Actually very few patients with both diseases have been described.

Overt thyroid disease was present in only case (C34) in whom a nodular goitre was present but whose thyroid function was normal clinically. A reaction with normal thyroid antigen to 1/10 was found in the complement fixation test, and the TCH (thyroglobulin) titre was 1/10240. Biopsy would have been valuable, and may well have shown thyroiditis. The tanned cell haemagglutination test for thyroglobulin antibodies was done in 24 cases of cirrhosis, 18 males

(3 positive) and 6 females (2 positive). This represents a frequency of positive reactions of 21%. 11 of these cases were included in the series documented in Table 6. In case C43 the titre was 1/10240 and a titre of 1/80 was obtained in the CFT (normal thyroid). The incidence of positive TCH reactions in 387 non-thyroid hospital patients was 1.6% (see p.).

This series is too small to show a moderate correlation, especially in women with cirrhosis.

THE A.I.C.F.T. IN ACUTE HEPATOCELLULAR DISEASE

This is a heterogeneous group. Sera were obtained from 10 cases of infectious hepatitis from Northfield Infectious Diseases Hospital and the Royal Adelaide Hospital. The diagnosis was made clinically on these cases.

The A.I.C.F. phenomenon was present in 2 of these 10 cases.

The rest of the cases documented in Table 7 were of acute or subacute liver disease of various aetiologies, except for the last two cases which were more properly classified as chronic cases of relapsing hepatic disease.

Case H.16 was diagnosed as having "chronic active hepatitis" with a recurrence of jaundice 2 months after the onset of a febrile illness with jaundice. Liver biopsy showed a chronic inflammatory condition with lymphocytic infiltration. The A.I.C.F.T. was negative.

Case H.17 had recurrent jaundice of unknown aetiology. At the time the serum bilirubin (direct and indirect Van den Bergh reactions) were estimated the patient was not jaundiced and normal figures were obtained. There was no evidence of haemolysis. The case therefore remains undiagnosed.

This patient had high titre A.I.C.F. antibodies, the highest being against liver antigen. Sections from a liver biopsy showed a lymphocytic infiltration, but the tissue was not black and did not contain the cytoplasmic granules found in the Dubin-Johnson syndrome.

A portion of this hepatic tissue used as antigen reacted with the patient's own and other sera, giving the same titres

as test antigens.

The other patient in this group having a positive A.I.C.F.T. (H.14) had a toxic hepatitis associated with lead poisoning, as a result of taking lead acetate as an abortifacient. The clinical manifestations were severe anaemia with punctate basophilia, jaundice, gross enlargement of the liver and moderate splenomegaly. The serum gave titres of 1/10 to kidney, adrenal, lung and thyroid, but not to liver. The A.I.C.F.antibodies were absent when the serum was tested six weeks after the patient left the hospital.

TABLE 8. A.I.C.F.T. IN ACUTE HEPATOCELLULAR DISEASE

Number	Positive with any antigen	Positive with			
		Liver	Kidney	Adrenal	Thyroid
17	5(29%)	2 (12%)	4 (23%)	3 (18%)	3 (18%)

The tanned cell haemagglutination (thyroglobulin) was carried out on 7 of these cases (4 female, 3 male) and was negative in all. It was also negative in 3 cases of acute hepatitis not included in this series.

TABLE 7. A.I.C.F.T. IN ACUTE HEPATOCELLULAR DISEASE

PATIENT	SEX	AGE	DIAGNOSIS	AICF				SIZE OF LIVER	COLLOIDAL GOLD	CEPHALIN COLEST.	$ZnSO_4$	ALB	GAMMA GLOBULIN	THYROID	TCH THYRO-GLOBULIN	
				LIVER	KIDNEY	ADRENAL	THYROID									
H1	F	50	Miliary T.B.	-	-	-	-	≠		3						Miliary tuberculosis involving liver (histological proof).
H2	M	41	P.A., hepato-megaly.	-	-	-	-	≠≠	5	4						Biopsy. Chronic non-specific inflammatory process.
H3	F	35	Infectious hepatitis	-	-	-	-	-	5	4	17.2				-	Kline positive, WR negative.
H4	M	22	Infectious hepatitis	-	-	-	-	-	0	0	9.3				-	
H5	F	?	Infectious hepatitis	-	-	-	-	-	-	-						
H6	M	53	Secondary carcinoma stomach.	-	-	-	-	-	-	-	9.3	3.69	0.96			Histological proof of liver metastases.
H7	F	21	Infectious hepatitis	-	-	-	-	≠								
H8	M	26	Infectious hepatitis, relapsing	-	-	-	-	≠	5	4	29.5					
H9	M	17	Infectious hepatitis	-	-	-	-									

TABLE 7 - (continued)

PATIENT	SEX	AGE	DIAGNOSIS	AICF				SIZE OF LIVER	COLLOIDAL GOLD	CEPHALIN COLEST.	$ZnSO_4$	ALB	GAMMA GLOB.	THYROID	TCH THYRO-GLOBULIN	
				LIVER	KIDNEY	ADRENAL	THYROID									
H10	M	64	Secondary carcinoma rectum	-	-	-	-	++				low	N		-	Raised alpha 2 globulin. Metastases in liver.
H11	F	27	Infectious hepatitis	80	80	80	80	+	5	4	20	4.61	2.03			
H12	F	42	Infectious hepatitis	-	-	10	-	+	5	4	35.5		2.45			
H13	F	30	Infectious hepatitis	-	-	-	-	-								
H14	F	26	Toxic hepatitis, (lead)	-	10	10	10	+++	0	3	13					
H15	F	20	Infectious hepatitis	-	-	-	-								-	
H16	F	50	Chronic active hepatitis	-	-	-	-	+		3		2.8	1.84			Probably followed infectious hepatitis. Liver biopsy showed chronic inflammatory cell infiltration.
H17	F	39	Idiopathic recurrent jaundice	160	80	80	80	±	3	4	19		+			Liver biopsy. Lymphocytic infiltration.

THE CONCLUSIONS TO BE DRAWN FROM THE STUDY OF THE 1014 CASES.

The conditions with which the phenomenon is significantly associated are:-

1. Syphilis and yaws.
2. "Collagen diseases".
3. Hepatic disease.
4. Thyroid disease.

Thus Mackay and Gajdusek's early findings are confirmed, and a major association with syphilis, and an association of thyroid A.I.C.F. with thyroid disease is revealed. The phenomenon was present in three cases of Waldenstrom's macroglobulinaemia or closely related states, absent in a case of globulin-producing lymphosarcoma, absent in 6 cases of myelomatosis, and present in a further case diagnosed myelomatosis which was not adequately studied or verified. Thus cases of paraproteinaemia other than those falling into the diagnostic category of myelomatosis are possibly associated with the phenomenon.

It was repeatedly found that the phenomenon was inexplicably negative in cases clinically identical to others where strong positive reactions occurred.

The A.I.C.F. antibodies are apparently stimulated to appear in the blood in a wide range of diseases. The incidence of positive reactions, when a positive result with any one or more antigens is accepted as a positive reaction is significantly greater in a group of persons sick with a variety of diseases than in ostensibly normal

blood donors. Titres represent the balance between antibody entering and leaving the circulation. Rapid fixation of antibody in the tissues may cause this anomaly.

In almost all instances more than one of the five antigens reacted; no pattern could be detected, however, in the spectrum of autoantibodies that was common to any condition or disease. Reaction occurred with thyroid antigen alone in some cases of thyroid disease.

With the exception of thyroid disease, there was no statistical correlation between the organ involved in the particular process and the antigen reacting.

Repeated tests on different samples of the same patient's serum, using antigens from different cadavers, showed a remarkable constancy of antibodies both with regard to the antigens with which they reacted, and to titre.

Mackay and Gajdusek found that the serological activity generally disappeared as patients with acute viral hepatitis recovered, except in the cases which became chronic. Only one patient with infectious hepatitis was followed up in this series, and in this case the autoantibodies also disappeared after three months.

The incidence of positive results was greater than 50% in only two conditions, viz. syphilis and systemic lupus erythematosus.

Analysis of the results by sex and age showed a higher incidence in females than in males below 60 years; above this the incidence was equal in the two sexes. The incidence of autoantibodies detected by these tests showed no clear tendency to rise or fall with increasing age.

There was a tendency, although the figures do not

attain statistical significance, for A.I.C.F. activity to be provoked by virus conditions other than infectious hepatitis. Conversely there was a lower incidence of antibodies in patients suffering from various bacterial infections.

This study therefore failed to establish a clinical criterion by which the presence of A.I.C.F. antibodies could be predicted. It confirmed Mackay and Gajdusek's findings and brought out a close correlation with the presence of Wasserman antibodies.

Muschel et.al. (1961) studied complement fixation by sera of humans and other species with extracts of calf thymus and rabbit liver. Many of the conclusions of this work were confirmed by these authors. They stated that most human sera with complement-fixing activity reacted similarly with antigens derived from human, rabbit and bovine livers. They were unable to induce the phenomenon experimentally in animals by x-irradiation, burning, and large amounts of various mixed vaccines (poliomyelitis, typhus, tetanus, diphtheria, plague, typhoid and paratyphoid).

Humans and animals did not generally develop these antibodies as a result of bacterial or viral infection. However, experimental infection of rabbits with trypanosomes caused the appearance of antibodies to rabbit liver and calf thymus. Absorptions with Wasserman antigen did not significantly reduce the tissue-antibody titre. Titres with liver and thymus paralleled the level of the Wasserman antibody, but there was no similar correlation of titre in

the Wasserman reaction with tissue-antibodies in sera of persons with biological false-positive Wasserman reactions.

In contrast to Muschel's findings Hackett and Beech (1960) showed the temporary appearance of A.I.C.F. antibodies in two of seven nurses during prophylactic immunization against poliomyelitis, tetanus, typhoid and paratyphoid fever.

The A.I.C.F. phenomenon is as baffling now as it was when it was discovered. The phenomenon is important not only because it is a mystery, but because it is apparently a manifestation of a bodily process of which we have little knowledge.

There is little collateral evidence to show that the A.I.C.F. antibodies are manifestations of an autoimmune process. It is reasonable and convenient to refer to these substances as antibodies since they behave as such in the classical technique used to detect them, and they are gamma globulins.

They may be true auto-antibodies in that they react in the complement fixation test with antigens derived from the patient's own tissues. However, Mackay and Larkin (1959) found that this was not always so. They reported the results of testing sera having A.I.C.F. activity from 11 patients against antigens from their own tissues. One of these patients was included as case H17 in the acute hepatocellular disease series of this paper.

In 7 of these patients there was partial or complete failure of the serum to react with autologous tissues despite high A.I.C.F. titres with homologous antigens, and organ extracts from the patients fixed complement

satisfactorily in combining with other reactive sera.

They were unable to account for this situation. The possibility of splenic removal of auto-antibody was not supported by experiments in vitro, in which it was not possible to remove A.I.C.F. activity by absorption with splenic tissue.

A natural experiment in the passive transfer of these antibodies provided good evidence that they do not cause tissue damage. A woman suffering from systemic lupus erythematosus, who had high titres of A.I.C.F. antibodies, was followed through pregnancy and the delivery of a healthy infant. The patient was first seen in the Royal Adelaide Hospital in 1954 at the age of 27 when she presented with acute haemolytic anaemia immediately after the birth of her first child (which has grown healthily). L.E. cells were found and steroid therapy instituted. Rheumatoid type arthritis, anaemia, neutropenia, splenomegaly and leg ulceration were present since that time, partially suppressed by cortisone. In 1959 she became pregnant again and was delivered of a healthy male infant. The A.I.C.F. antibodies of mother and infant are summarized below.

TABLE 9. A.I.C.F. ANTIBODIES IN MOTHER WITH S.L.E. AND CHILD.

	At birth		6 weeks after birth	
	Mother	Child	Mother	Child
Liver	160	10	160	10
Kidney	160	10	160	10
Adrenal	160	10	160	10
Thyroid	160	10	160	10

Figures are reciprocals of titres.

The L.E. cell phenomenon was present in the infant's blood at birth, and rosettes were present at six weeks. The child's progress when last seen at three months was satisfactory.

There have been several other cases of unaffected children being born to mothers with systemic lupus erythematosus (Holman, 1960).

If exposure of developing foetal tissues to these autoantibodies causes no damage it is most unlikely that they mediate disease processes.

Asherson and Broberger (1961) have shown antibodies to antigens prepared by hot phenol-water extraction of fresh human colon and liver in the serum of patients with liver disease, rheumatoid arthritis, the nephrotic syndrome, and ulcerative colitis. These antibodies were apparently distinct from A.I.C.F. antibodies and were demonstrated by tanned-cell haemagglutination. Their summary of current hypotheses is perhaps the best to date.

Auto-antibody formation may result from abnormality of the antibody-producing system, or from an abnormal or unusual antigenic stimulation or a combination of both. The abnormality in systemic lupus erythematosus may be in the antibody-forming system. Burnet (1959) suggested that there is a mechanism by which the development of clones of auto-aggressive R.E. cells from mutant cells is checked. Unusual antigenic stimulation may occur in the following situations:

(1) Release of potentially antigenic body constituents to which tolerance had not been acquired.

(2) Loss of tolerance to normal tissue components.

(3) Alteration of a normal tissue component rendering it antigenic and causing the formation of antibody cross-reacting with a body constituent.

(4) Attachment of a normal tissue component (Hapten) to a carrier which renders it antigenic.

(5) Exposure to foreign antigens possessing determinants similar to those of some body constituent(s), causing the formation of antibody cross-reacting with this body constituent.

Grabar (1958) suggested that these antibodies are protective rather than auto-aggressive and function as transport globulins. The A.I.C.F. phenomenon in such a hypothesis is to be thought of as a manifestation of a physiological process. These antibodies may constitute a homeostatic mechanism which operates when various stresses are altering the metabolism of parenchymal cells of the body. Possibly one or more metabolic steps are rendered inefficient, with a resultant build-up of metabolites which escape from the cells at such times, and stimulate antibody production.

Any physiological process is liable to exaggeration and distortion as a result of disease, and disease may secondarily result from disturbance of a physiological mechanism. This process is disturbed in syphilis, systemic lupus erythemastosus and liver disease, but also in persons suffering from a wide variety of apparently unrelated maladies. A similar phenomenon is normally present in a high proportion of rabbits (Kidd and Friedewald, 1942) although the auto-antibody in this case is heat-labile. Heat-stable complement-fixing auto-antibodies can be

produced in rabbits by immunization with rat liver in Freund's complete adjuvant (Asherson and Dumonde, 1962). Natural antibodies were also found in the serum of monkeys, chimpanzees and rats (Muschel et al, 1961).

Identification of the antigens with which the A.I.C.F. antibodies react is necessary for progress in understanding the phenomenon. Asherson (1959) showed that the sera of some patients with systemic lupus reacted with desoxyribose nucleic acid as well as with soluble and insoluble cytoplasmic protein of the rat liver. Sera from patients with other diseases reacted with the soluble and insoluble cytoplasmic proteins, but not with nuclear constituents.

The A.I.C.F. phenomenon has been cited as evidence of an auto-immune pathogenesis of cirrhosis of the liver by Mackay (1958, 1960; Mackay, Taft and Cowling, 1956), Havens (1959) and others. There are several other tenuous pieces of evidence to support this suggestion; e.g. that some cirrhotics form very high levels of specific antibody (Havens, Shaffer and Hopke, 1951), although this evidence has been challenged by Cherrick et al (1959); there is generally a raised gamma globulin level; the mean half-life of I^{131}-labelled gamma-globulin in cirrhotics is reduced from 14 to 9 days (Havens et. al., 1954), so that high levels must be achieved by a considerable increase in the rate of synthesis; the finding of plasma cells and increased numbers of Kupffer cells in the cirrhotic liver (Havens, 1959); the finding of L.E. cells in some cases of cirrhosis, particularly in young women (Joske and King, 1955; Bearn, Kunkel and Slater, 1956).

Gear (1955) postulated that the combination of liver

TABLE 10. (after Hughes, 1933).

	Immunization	Animals	Serum
1.	Yellow fever infected monkey liver	Yellow fever vaccinated monkeys	Precipitin reaction with yellow fever infected liver or normal liver.
2.	Normal monkey liver	Yellow fever vaccinated monkey	No serological reaction.
3.	Serum hepatitis	Human	A. Precipitin with monkey serum containing antibody produced in (1). B. Precipitin reaction with yellow fever infected monkey serum.
4.	Yellow fever	Monkey	Precipitin reaction in convalescent serum with normal monkey and other animal liver extracts.
5.	Serum hepatitis	Human	Precipitin reaction as above.

cell and virus functions as an auto-antigen, and that the subsequent union of antibody and antigen resulted in damage to hepatic cells, which in turn led to further release of auto-antigen. Stimuli other than viruses might initiate the process. He showed that neo-arsphenamine-damaged rabbit liver, but not normal liver injected into rabbits caused production of precipitating and complement-fixing antibodies. Gear cited the work of Hughes (1933) in support of his theory (see Table 10).

Behar and Tal (1959) produced hepatic necrosis in some guinea pigs and hamsters by injections of homologous liver homogenate in Freund's adjuvant. This has not been confirmed, although Steiner et al (1960) found hepatic lesions in guinea pigs injected with mixtures of homologous or autologous adrenal tissue with Freund's adjuvant. These authors suggested that adrenal and liver tissue components may share common or closely related antigenic determinants responsible for the cross reactivity.

Finally Weir (1961) showed that rats developed A.LC.F.-type antibodies rapidly after treatment with carbon tetrachloride, rising to maximum titres at 3 or 4 days, and falling off rapidly in the next 3 or 4 days. He also found that the serum of one of the 12 control rats fixed complement with rat liver homogenate to a titre of 1/64. It has recently been shown that liver damage caused by carbon tetrachloride (Rees, Sinha and Spector, 1961) and bacterial endotoxin (Gordon, 1961) results in release of liver-cell components into the blood-stream.

In summary, therefore, there is altered activity of the lymphoreticular system in cirrhosis and other liver

diseases, with the production of auto-immune antibodies, but no proof of an immunological pathogenesis of liver disease.

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Reprinted from the BRITISH MEDICAL JOURNAL, *July* 2, 1960, *vol. ii, pp.* 17–20

AUTOIMMUNE COMPLEMENT-FIXATION REACTION IN 1,014 PATIENTS

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Gajdusek (1957, 1958), and Mackay and Gajdusek (1958) reported that fixation of complement occurred when sera from certain hospital patients were incubated with simple saline extracts of various human organs. They called this the "autoimmune complement-fixation reaction" (A.I.C.F.). It had no strict organ specificity, and it occurred most often in cases of liver disease, diffuse "collagen" disease, and macroglobulinaemia. Gajdusek (1958) gave his reasons for regarding the reaction as one between antibody and antigen, and for adopting the view that it is an "autoimmune" phenomenon.

We have looked for this A.I.C.F. reaction in the sera of 1,014 medical patients in the Royal Adelaide Hospital, and report the results, together with a discussion of some possible interpretations of the reaction other than on a basis of autoimmunity.

Materials and Methods

Source of Sera

Blood was taken as a routine from patients admitted to the beds of the professorial unit of the Royal Adelaide Hospital. Other sera were obtained from time to time from large groups of patients in other medical clinics of the hospital. A few specimens were taken selectively from cases of syphilis, yaws, cirrhosis, diffuse "collagen" disease, or from cases known to have abnormal serum protein electrophoretic patterns. Usually only one test was made on each patient, and this was soon after admission or in an acute phase of the disease, when diagnosis or management was under active consideration. Every one of the 1,014 cases was later reviewed and a diagnosis assigned without reference to the A.I.C.F. results, which were recorded separately.

Complement-fixation Test

Complement.—Pooled guinea-pig serum was stored in small amounts at −20° C.

Antigens.—Fresh organs, obtained surgically or at necropsy, were homogenized to a 10% suspension in physiological saline in a Waring blender, then centrifuged three times at 3,000 r.p.m. for 10 minutes at +4° C. and the sediments discarded. The supernatant solution was the antigen, and was stored in small containers at −20° C. for up to three months. Antigens were prepared from normal liver, kidney, lung, adrenal, and thyroid gland.

Diluent. — Calcium - magnesium saline was used throughout the test (Mayer *et al.*, 1946).

Technique.—A five-volume technique, using 0.2-ml. volumes in Wassermann tubes, was employed. Sera were inactivated for 30 minutes at 56° C. before testing. Serum dilutions, antigen, and 2½ HD_{50} of complement, titrated previously in the presence of antigen, were allowed to stand overnight at +4° C. and then for one hour at bench temperature. Two volumes of haemolytic serum were then added to each tube and incubated at 37° C. for 30 minutes, after which the cells were sedimented by light centrifuging. 50% haemolysis was taken as the end-point. Each test was accompanied by a serum anticomplementary control, and known positive and negative sera were included in each batch of tests. Sera were initially screened at 1/10 and 1/80, and then titrated fully if complement-fixation occurred. A "positive result" was complement-fixation at 1/10 or a higher dilution. There were 10 sera among the 1,014 which were anticomplementary at 1/10 without antigen, and we regarded these as giving a negative A.I.C.F. reaction.

Results

The results are given in Table I, from which it is clear that many sera fixed complement with several antigens, rather than with one only; in fact, this was the rule and not the exception. Neither a characteristic pattern of activity with a group of antigens nor any reciprocal relationship between antigens could be seen on an ordinary inspection of the results, except that, of the five antigens used, kidney and liver were most regularly among the positive reactors. (Liver, kidney, adrenal, and thyroid antigens were used to test all the 1,014 sera, but only 903 of them were tested with lung. Consequently the appropriate reduced total of cases tested with lung is shown in each disease category in Table I.)

The allocation of patients to groups of diseases in Table I was made after a review of every case and without reference to the serological results. The association of diseases within the groups are apparent in most of them. Group 4 is one of the loosest, comprising the theme of vascular insufficiency in a broad way to include diabetes (with its arteriosclerotic complications) and haemorrhage, as well as cardiac and cerebral infarction, but not periarteritis nodosa, which is in group 7. Viral hepatitis is in group 8. Group 10 shows separately some conditions which may be of interest from the "autoimmune" point of view, but the bulk of this group is made up of undiagnosed cases, with some miscellaneous examples of comparatively rare conditions not classifiable under any of the other heads.

It is clear from an inspection of the table that the incidence of a positive A.I.C.F. reaction in the whole

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group of 1,014 hospital medical patients is much higher than in 164 apparently healthy blood donors, and that Gajdusek and Mackay's findings in liver diseases, "collagen" diseases, and paraproteinaemia are generally confirmed. Wishing to make useful comparisons between groups of people with different diseases (and not merely between disease and the healthy state), we decided that group 10 represented a suitable control collection of persons who were sick to a similar degree, being admissible to the same wards over the same period in the same hospital under the same physicians, as those in the other groups of more readily defined disease states. Consequently we have made a comparison between group 10 and each other group, using the χ^2 test, of the incidence of the A.I.C.F. reaction between the patients' sera and each antigen in turn. Those which vary significantly (for the numbers observed) from group 10 are shown in Table II.

The thyroid gland is the only organ which shows a good correlation between A.I.C.F. activity and clinical disease in the same organ as that from which the antigen derives. We are reporting on this elsewhere (Hackett *et al.*, 1960).

Some virus conditions other than viral hepatitis, particularly infectious mononucleosis, though not necessarily with a positive Paul–Bunnell test, seem to provoke A.I.C.F. activity. The positive reactions obtained in syphilis and yaws are not unexpected when it is considered how similar the A.I.C.F. technique is to the Wassermann reaction.

Group 4 and group 6 show respectively a possible deficiency of anti-kidney and anti-lung activity compared with group 10, but at a low ($P=0.05$) rate of significance.

TABLE II.—*Probability (P) Values for the Statistically Significant Differences Between the Positive Results in Various Groups from Table I compared with Group* 10

Group	Any One of the 5 Antigens	Liver	Kidney	Adrenal	Thyroid	Lung
1	<0·001	<0·001	<0·001	<0·001	<0·001	
3			0·05	<0·01	<0·05	
4			0·05			
6						<0·05
7	<0·01	<0·01	0·02	0·001	<0·001	<0·01
8	0·001	0·02	0·001	0·001		<0·001
9	<0·01		0·05		<0·002	

TABLE I

Group	Disease	Total Tested with Liver, Adrenal, Kidney, Thyroid	Positive with any of these Antigens, or with Lung		Positive with Liver		Positive with Kidney		Positive with Adrenal		Positive with Thyroid		Total Tested with Lung	Positive with Lung	
			No.	%	No.	%	No.	%	No.	%	No.	%		No.	%
1	Syphilis and yaws (W.R.+)	15	13	*87*	9	*60*	8	*53*	12	*80*	11	*73*	4	2	*50*
2	Bacterial infections	68	7		4		5		4		1		65	3	
	Tuberculosis	11	2		2		0		0		1		11	1	
	Total	79	9	*11*	6	*8*	5	*6*	4	*5*	2	*3*	76	4	*5*
3	Viral infections	53	13	*25*	11	*21*	13	*25*	9	*17*	11	*21*	53	7	*13*
4	Diabetes	21	3		3		2		2		2		21	2	
	Peripheral vascular disease, Raynaudism	8	1		0		0		1		0		8	0	
	Atherosclerosis, coronary artery dis., infarcts of heart and brain, cerebral haemorrhage	80	9		5		5		5		5		79	4	
	Peptic ulcer, gastro-intestinal haemorrhage	33	5		3		2		3		4		30	2	
	Total	142	19	*13*	11	*8*	10	*7*	11	*8*	12	*8*	138	9	*7*
5	Carcinoma, sarcoma	176	28	*16*	14	*8*	19	*11*	14	*8*	10	*6*	133	12	*9*
6	Reticuloses	37	9		4		5		4		1		22	0	
	Lymph. leukaemia	10	2		1		2		2		0		10	0	
	Myel. ,,	10	2		1		2		1		0		10	0	
	Myeloproliferative disorders	15	0		0		0		0		0		15	0	
	Para- and dys-proteinaemia	12	4		2		4		2		3		12	2	
	Total	84	17	*20*	8	*10*	13	*15*	9	*11*	4	*5*	69	2	*3*
7	Dissem. L. E.	13	10		8		8		7		9		12	7	
	Idiopathic acquired haemolytic anaemia	6	3		3		2		2		2		5	3	
	Rheumatoid arthritis	31	10		4		7		5		1		24	3	
	Rheumatic fever	18	1		0		1		0		1		18	0	
	Other diffuse "collagen" diseases	15	4		4		3		3		9		13	3	
	Total	83	28	*34*	19	*23*	21	*25*	17	*20*	22	*27*	72	16	*22*
8	Cirrhosis	60	21		14		19		13		8		56	14	
	Acute hepatocellular disease	18	6		3		5		4		4		16	6	
	Total	78	27	*35*	17	*22*	24	*31*	17	*22*	12	*15*	72	20	*28*
9	Thyroid diseases	69	24	*30*	11	*14*	16	*20*	8	*10*	16	*20*	73	10	*14*
10	Glomerulonephritis	15	3		3		3		2		2		13	1	
	Asthma	19	1		1		1		1		1		17	1	
	Hypersensitivity, allergy	5	0		0		0		0		0		5	0	
	Deficiency anaemias	19	3		0		1		1		1		19	0	
	Addison's disease	2	0		0		0		0		0		0	0	
	Nephrotic syndrome	6	1		0		1		1		1		6	0	
	Miscellaneous and undiagnosed	169	31		21		24		9		16		153	18	
	Total	235	39	*17*	25	*11*	30	*13*	14	*6*	21	*9*	213	20	*9*
	Grand total	1,014	217	*21*	131	*13*	159	*16*	115	*11*	121	*12*	903	102	*11*
	Healthy blood donors	164	13	*8*	8	*5*	9	*6*	8	*5*	3	*2*	164	6	*4*

An analysis by sex and age was made of 988 of the sera from patients where details of the age could be satisfactorily established. The results showed a higher incidence in females than in males in all decades below 60 years; above this the incidence was equal in the two sexes. The percentages are given in Table III. There was no clear tendency for the incidence of the A.I.C.F. reaction to rise or fall with increasing age in either sex.

We found that not one of the following blood-group antigens was exclusively present or absent in the red cells of all of 10 positive A.I.C.F. reactors taken at random: A_1, A_2, B, O; C, c, D, d, E; Le(a), Le(b); M, N; Fy(a); Jk(a). All 10 were e-positive and Kell-negative, but this would be so with over 90% of the patients in the wards.

TABLE III.—*Age Incidence of the A.I.C.F. Reaction Shown as the Percentage of Positive Results in 455 Male and 533 Female Patients*

Percentage Positive	Age Groups (Years)					
	0–40		41–60		61–100	
	M	F	M	F	M	F
With any of the five antigens	15	22	13	24	21	21
With liver	9	13	9	13	12	13
,, kidney	11	17	9	17	17	16
,, adrenal	11	13	8	10	9	11
,, thyroid	7	14	7	10	8	10
,, lung	6	15	5	14	14	11
Total No. in each group	138	149	172	189	145	195

We found, as did Gajdusek, that the antigenic activity of the extracts was destroyed by heating to 60° C. for 30 minutes. Unlike him, we found that the activity was not removed by centrifuging at 65,000 *g* for 120 minutes, and so we have concluded that at least some of the antigenic activity in our preparations resides in soluble components smaller than cell microsomes.

Discussion

These findings have confirmed the clinical correlations found by Gajdusek and Mackay. We believe they were right to interpret this "A.I.C.F. reaction" on an "autoimmune" basis. The prevailing climate of opinion suggests that autoimmunity in some auto-aggressive form may be the underlying mechanism in many diseases, particularly some in which this reaction is frequently observed. At the same time there are other possible interpretations which we now consider.

Lipid-lipid Interactions, and Anti-lipid "Biological" Reagins

The lipid levels found in plasma can vary physiologically from one individual to another, between the two sexes, and pathologically in conditions such as hepatocellular disease, nephrosis, diabetes, and atheroma. This applies to characterized lipids such as cholesterol, cephalin, and lecithin, but the same may be true, and for other pathological conditions, of various fats, fatty acids, steroids, bile acids and salts, phospholipids, glycolipids, and lipoproteins.

All cells contain lipids, and the simple "antigen" extracts made for the A.I.C.F. reaction are rich in derivatives from broken cells. If certain tissues have peculiar combinations of lipids these would probably be characteristically represented in the extracts.

The Wassermann reaction (and similar complement-fixation tests for syphilis) is a refined prototype of the A.I.C.F. reaction in the sense that an original simple liver extract has now been replaced by purified cardiolipin. Such reactions are very sensitive to the addition of other lipids, so that a mixture of lipids can show some special activities *in vitro* which its individual components will not. Rapport *et al.* (1959) use the phrase "lipid-lipid interactions" to describe this.

The "biological" false-positive complement-fixations encountered in non-treponemal disease can now be identified by showing that the sera lack antitreponemal activity in the immobilization test. But Kent *et al.* (1958) have shown that such false-positive reactions can be distinguished from true syphilitic ones in ordinary complement-fixation or flocculation tests with cardiolipin antigen, by adding specific amounts of lecithin which at different concentrations lead to a selective reactivity of either the syphilitic or the non-syphilitic anti-lipid. Such an observation makes a valuable practical distinction but does not elucidate the nature of the "biological" anti-lipid reagent. Nevertheless, it suggests how the A.I.C.F. reaction, with its lipid or lipid-rich antigens and probable sensitivity to other lipids in the serum, might depend on a reagent of the same type, and show non-immunological variation in complement-fixation among sera from patients with plasma-lipid abnormalities.

Eastwood (1920), in a review of early experimental work on the nature of the Wassermann reaction, showed how much of this was based on the opinion (which it appeared to support) that the syphilitic reagin was not an antibody arising *de novo*, but a constituent of normal plasma which was physically altered in syphilis and thereby changed from a previously masked state. Mackie and Watson (1926) and Mackie and Finkelstein (1928) showed that the Wassermann reagin was a serum constituent which could be found in heat-sensitive form, being destroyed at 55° C. in many, if not all, normal human sera, but was characteristically heat-stable at 55° C. in the sera of rabbits and certain other animals. In human syphilis it seemed to acquire a heat-stable form so that it survived the "inactivation" or heat treatment (56° C. for 30 minutes) that always preceded the serological tests. Kahn (1950) claimed that a heat-stable "syphilitic" antibody could be found in nearly all human sera if a sufficiently delicate technique was used. If the foregoing is true, the role of this specific globulin reagent (heat-sensitive or otherwise) in the normal body must be considered. If it is present in all people, and is only multiplied or altered physically by disease in some way which does not change its immunological specificity, then it cannot be looked on as an autoaggressive agent; it must have a physiological function, even if the term "autoantibody" is used to describe it.

Kidd and Friedewald (1942) have shown that heat-stable complement-fixing antibodies reacting with simple saline extracts of various organs, particularly liver and kidney, can be found in most rabbit sera, and we have confirmed this. Most rabbits, even though bred in captivity, are not diseased according to current concepts of pathology, so here is an indication that the presence of A.I.C.F.-type antibodies is a normal feature in some vertebrates. If the same were true of man, variations in heat stability combined with lipid sensitivity as outlined above could produce many of the patterns shown by the A.I.C.F. reaction in health and disease without invoking acquired autoimmunity. We are at present planning to investigate the effects of adding lecithin or cholesterol to the A.I.C.F. reaction, and of using complement-free unheated sera.

Enzyme Activity

Extracts used as antigens in the A.I.C.F. reactions must contain cell enzymes. Beeson and Rowley (1959) have shown that kidney has anticomplementary activity, probably because renal glutaminase liberates ammonia which destroys the fourth component of complement. If the sera of certain sick people contained relevant coenzymes or activators, it is conceivable that an apparently specific removal of complement might occur when serum and " antigen " were mixed. This is another possible non-immunological explanation of the A.I.C.F. reaction. It could be investigated by the use of enzyme inactivators.

Aggregated Globulins

The lists of diseases in which the A.I.C.F. reaction is most commonly positive are similar to those in which " biological " false-positive complement-fixation tests for syphilis are most frequently found (Moore and Mohr, 1952), and are also similar to those where positive results in non-rheumatic conditions are obtained with ultra-sensitive elaborations of the Rose–Waaler test such as the latex-fixation reaction (Dresner and Trombly, 1959). There are indications that such reactions all detect a similar feature in serum which is the presence of abnormal large-molecule plasma globulins, perhaps produced by simple aggregation of smaller normal globulins. The tendency to produce these macro-globulins may be inherited, and be more common in women than in men ; it may be accentuated by chronic hepatocellular disease, and may predispose to arthritis and the development of other symptoms associated with diffuse " collagen " disorders. Moore and Lutz (1955) produced evidence showing that in women a " biological " false-positive test for syphilis was often followed within a few years by the development of disseminated lupus erythematosus. This is in accord with the view of Haserick (1955), whose clinical studies led him to conclude that there was a " diathesis " or predisposition to this disease.

Autoaggressive or Not ?

If the A.I.C.F. reaction is due to the activity of a macroglobulin or to that of a normal or " natural " reagin (which in the light of this discussion may amount to the same thing), the question remains whether or not it is an antibody–antigen reaction. It may not be, if all true antibodies arise only after classical " non-self " antigenic stimulation. However, if patterned globulins can arise without specific antigenic stimulation in some such way as Burnet (1959) visualizes, through the function of a special or uncontrolled clone of cells, then the reaction can be looked on as the equivalent of one between antibody and antigen in a broad sense, and must be regarded as playing a possible autoaggressive part in the aetiology of the diseases with which it is associated. However, an autoaggressive role for such globulins is not the only possible one, and on present evidence is not substantiated. " Autoantibodies " can sometimes be found in apparently healthy people. There may even be physiological patterned antibody-like globulins which play a normal part in the body's economy, and if this is not kept in mind their activity *in vitro*, in reactions like the A.I.C.F., may wrongly be taken as good evidence for the existence of an aggressive autoimmune process.

Summary

The sera of 1,014 mixed hospital medical patients have been tested for the " autoimmune complement-fixation " (A.I.C.F.) reaction described by Gajdusek (1957, 1958), using simple saline extracts of normal liver, kidney, lung, adrenal, and thyroid gland as antigens.

Some positive reactions were found in most common diseases, but the clinical correlations noted by Mackay and Gajdusek (1958) have been confirmed in that positive reactions, usually with more than one antigen, were found with significantly greater frequency in the sera of cases of liver disease, " collagen " disease, and paraproteinaemia ; and also in syphilis and yaws, some viral conditions, and thyroid disease. The thyroid was the only organ which showed some correlation between regular complement-fixation with a particular organ extract and clinical disease in the same organ.

Autoimmune interpretations of the A.I.C.F. reaction are valuable, but others which are discussed above should be kept in mind. These are the possibilities that the effect is a lipid-lipid interaction, or an enzymic artifact, or a phenomenon produced by the practice of heating sera to inactivate them before complement-fixation tests are performed, or that it represents a non-immunological alteration in specific globulins.

We are grateful to Professor Robson and the honorary physicians of the Royal Adelaide Hospital who sent us blood samples and allowed us access to their cases. Dr. Kathleen Maros helped us to check and annotate more than a thousand sets of case notes. Mr. Vincent, of the Red Cross Blood Transfusion Centre in Adelaide, kindly did some special blood-grouping. Miss N. Deering and Miss B. Rainsford gave valuable technical assistance.

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AUTO-IMMUNITY IN THYROID DISEASE

CONTENTS

CONTENTS
(continued)

AUTO-IMMUNITY IN THYROID DISEASE

Accumulation of plasma cells and lymphocytes, elevated levels of gamma globulin, and auto-antibodies in the blood are evidence of involvement of the reticuloendothelial system in Hashimoto's disease. An analogous condition can be produced in experimental animals. In addition it is one of a small group of conditions for which there is a plausible hypothesis to explain why the reticuloendothelial system might react to thyroglobulin as a foreign substance.

Burnet (1959) stated "This (Hashimoto's disease) is the only condition in human pathology that has been clearly shown to be an auto-immune condition in the sense of being a result of the production of antibody by normal body cells against a definable antigen".

At the time Burnet wrote these words some criteria stated at the beginning of this thesis had been satisfied. One of the antigens, thyroglobulin, had been partially characterized, and antibodies to this substance had been demonstrated in a high percentage of cases of Hashimoto's disease. An analogous condition had been induced in experimental animals, and circulating antibodies of similar type were commonly present in these animals. Thus Witebsky's four criteria have been met, and so there is good evidence for regarding Hashimoto's disease as an auto-immune condition. However, in accordance with the further strict criterion enunciated in this thesis, the case has not been proved conclusively. The lesions of Hashimoto's disease

have not been reproduced by transfer either of antibodies or cells bearing antibodies to thyroid tissue.

History

Recognition of elevated serum gamma globulin in four patients with Hashimoto's disease by Fromm et al. (1953), and subsequently of abnormalities in serum flocculation tests in this condition (colloidal gold, thymol turbidity and cephalin cholesterol flocculation tests) (Luxton and Cooke, 1956; Skillern et al. 1956), suggested an immunological disturbance.

Roitt, Doniach, Campbell and Hudson succeeded in demonstrating precipitating antibodies to thyroglobulin in Hashimoto's disease in 1956. Also in 1956 Rose and Witebsky reported the production of lesions resembling those of human Hashimoto's disease in rabbits by immunizing them against extracts of rabbit thyroid by the following method: one lobe of a rabbit's thyroid gland was removed and from it thyroglobulin was prepared, mixed with Freund's adjuvant, and injected into the same animal's footpad. After an interval of about three weeks antibodies were found in the blood of most of the rabbits tested by the tanned cell haemagglutination technique of Boyden (1951). A month later the remaining lobe of the thyroid was removed and sectioned. Lesions were frequently found. There was lymphocytic infiltration and glandular destruction, changes very similar to the histological changes in Hashimoto's disease. There was not, however, an absolute correlation between the occurrence of lesions and the presence of antibodies.

The thyroiditis first produced by Witebsky's group (Witebsky

et al. 1957) was caused only by homologous thyroid tissue. Subsequently experimental disease was produced by heterologous immunisation in rabbits by giving intravenous injections of hog thyroglobulin over a prolonged time. Complement fixing and precipitating antibodies against rabbit thyroid extract also appeared (Witebsky, 1958). Absorption experiments showed three types of antibody in rabbit anti-hog thyroid serum. Some antibodies reacted only with the thyroglobulin used for immunization, others cross-reacted with certain thyroglobulins from other animals (e.g. beef) and a small part reacted with thyroid antigen from the rabbit itself.

Witebsky (1958) summarized the properties of the circulating antibodies in experimental animals in which thyroiditis had been induced by immunological techniques.

1. Demonstrable by tanned cell haemagglutination.
2. Precipitation demonstrable, in favourable cases, either in fluid media or in gel using the Oudin or Ouchterlouy methods. The precipitate is soluble in excess antigen and a sharp equivalence zone can be established where neither antigen nor antibody are demonstrable in the supernatant after removal of the precipitate itself.
3. Fixation of complement occurs, again with a characteristic curve showing inhibition in zones of antigen excess.
4. Passive cutaneous anaphylaxis demonstrates antibody.
5. Antibody-antigen combination is also demonstrated by the fluorescent antibody technique of Coons.
6. Immunized rabbits give direct skin tests of the delayed type.

The antibodies detected by complement fixation and tanned cell haemagglutination tests are doubtless directed towards different antigenic components of the thyroid gland, since they also do not correlate exactly.

The serum factors have other characteristics of antibody according to Witebsky; migrating in or very near the gamma globulin fraction and "more or less" stable when heated to 56°C. for 30 minutes.

Jones and Roitt (1961) produced thyroiditis in rats using sheep thyroglobulin. They also produced rat thyroiditis with homologous thyroid extract. The lesions in the latter experiments showed two interesting features: the onset was rapid -- within 10 days, and the lesions disappeared after a year or less despite continued immunization.

Terplan et al. (1960) summarized the results of six years' experimental work in producing thyroiditis in rabbits, guinea pigs and dogs by one or more intradermal injections of homologous saline thyroid extract. Heterologous extract was used to cause thyroiditis in a few rabbits only.

The severity of the lesions did not correlate well with the level of circulating haemagglutinating, complement fixing and precipitating antibodies. Localization of gamma globulin in the colloid was also shown by the fluorescent antibody technique in some cases. Immunization of rabbits with hog or dog thyroid extract caused the appearance of antibodies far more frequently than thyroiditis.

Thyroiditis was produced with difficulty in dogs, and the lesions were found in 5 dogs in whose blood no antibodies were

detected. Conversely, lesions were always present when antibodies were found. Cross circulation experiments with 5 dogs showed that passive transfer of antibody did not cause lesions, and the transferred antibody disappeared rapidly.

No lesions were found in 5 rabbits, in which antibodies had not developed in the blood. Antibody was present in 7 animals in which the thyroid was histologically normal. When repeated injections were given the time factor and the number of injections seemed to have no influence on titre level or on the extent of histological alteration. Thyroiditis occurred in 45 out of 57 injected rabbits. Injection into 29 rabbits of heterologous tissue from different sources resulted in mild lesions in 10 animals, and a further 12 animals receiving normal, colloid nodular and cancerous human thyroid extract no thyroiditis was seen. A curious, foamy change was seen in the epithelium of several of the rabbit thyroids who had received heterologous injections. In all of the guinea pigs injected there were slight or moderate lesions, often unaccompanied by serum antibodies.

Since the work of Roitt, Doniach, Campbell and Hudson, and Rose and Witebsky in 1956 retrospective scrutiny of the literature has brought forgotten work to light. The subject has been reviewed frequently (Brit. med. J. 1958; Owen 1958; Zieve 1959; Robbins and Rall 1960; Hall 1962).

The significant papers are summarized:

1908 Marinesco. Positive C.F.T. between aqueous extract of toxic thyroid gland and serum of patients from whom the glands were taken.

1911 Papazolu (student of Marinesco). C.F.T. in 26 of 38 patients with Basedow's disease, using toxic thyroid extracts, and no C.F.T. in normal controls.

1915 Beebe. Treatment of Graves' disease with thyroid antiserums "achieving a modicum of success".

1927 Hektoen, Fox and Schulhof demonstrated precipitating antibodies in the serum of a rabbit which had received injections of the thyroid extract of another species. These antibodies reacted with thyroid extracts from the dog, goat, guinea pig, horse, rabbit, sheep, deer, racoon and zebra, but not with other tissues. Several workers have confirmed this lack of species specificity, but Adant and Spehl (1934) could not wholly confirm it. The anti-thyroid gamma globulins found in Hashimoto patients by Roitt and Doniach (1957) did not react with thyroglobulin from rabbit, rat, sheep, pig, cow or horse. However, thyroglobulin from Rhesus monkey or the chimpanzee cross-reacted with the human antibody (Owen, 1958).

1933 Ferguson showed that colloid extracts injected subcutaneously caused a giant-cell inflammatory reaction with epithelioid cells similar to the tuberculous reaction.

1942 Lerman injected human thyroglobulin into rabbits and detected circulating precipitins to rabbit thyroglobulin. Myxoedema occurred in the immunized rabbits in a few instances.

1959 Cline et al found no cross reactions with thyroid extracts from rabbits or rats, or pork thyroglobulin, when using the haemagglutination test with serum of patients with chronic lymphoid (Hashimoto's) thyroiditis, but serum from rabbits

immunized with extracts of normal human thyroid tissue reacted with thyroid extracts from many species.

It appears therefore that hetero-immunization produces antibodies which react with thyroglobulin from a broad range of animal species, whereas iso-immunization produces antibodies with a narrow range of reactivity, confined to the species and possibly to closely related species.

THE NATURE OF THE ANTIBODIES AND ANTIGENS IN THE SEROLOGICAL REACTIONS OF THYROID DISEASE.

The antibodies in human serum which react with thyroglobulin in precipitation reactions are found in the gamma-globulin fraction on electrophoretic analysis (Roitt, Campbell and Doniach, 1958). Gamma globulin of Hashimoto sera yielded the only fraction reacting with thyroglobulin to give precipitating reactions. Antibodies were demonstrable only in the gamma globulins of pathological sera. The identity of the fractions obtained by electrophoresis was confirmed by immuno-electrophoresis. An extremely high level of circulating antibody is present in some cases (as much as 5 mg./ml. of serum) whereas precipitin levels rarely exceed 2 mg. of antibody protein/ml. in the human after immunization with foreign proteins (Roitt, Campbell and Doniach, 1958).

Sera from some cases of Hashimoto's disease give a "rabbit precipitin" curve, in which the antigen is completely precipitated in the region of antibody excess. Other sera give a "horse flocculating" type curve in which the antigen is soluble in the region of antibody excess. Multiple (at least 3) lines in agar gel diffusion precipitation tests indicate that more than one antigen in the colloid takes part in the precipitin reaction.

Goudie, Anderson and Gray (1959) have described a non-precipitating antibody which occurred in one of their patients with histologically proven Hashimoto's disease. This antibody

to thyroglobulin formed a clear line instead of the usual white line of precipitation in an Ouchterlony agar gel diffusion system. Their studies revealed the following properties:-

1. That the reaction had the same specificity as the usual precipitin line formed by antigen-antibody reaction.
2. The serum exhibited inhibiting and enhancing effects in agar and in fluid media with selected precipitating Hashimoto sera.
3. The staining properties of the clear line indicated that it coincides with a zone of high protein concentration similar to that found in the usual opaque white precipitate formed by other Hashimoto sera.
4. The serum gave an exceptionally high titre in the tanned cell haemagglutination test.

Goudie et al. stated that most of the properties of this antibody have been described previously in different contexts. To explain the results of their experiments they suggested that the serum contained potentially multivalent antibody which for unknown reasons did not precipitate with homologous antigen. The serum after storage for some time gave the usual type of reaction of Hashimoto serum, a white precipitin line, which indicates the presence of a multivalent antibody. Hence their term "potentially multivalent". The enhancing effects of this serum when added to certain sera incapable of giving a precipitin reaction led these authors to postulate that some sera containing mixed antibody types may be able to give double precipitin lines with a single antigen, contrary to the usual belief that double

lines indicate two similar but separate antigens. If the serum contains a mixture of antibodies of "horse" type which react with different sites on the antigen, a second line may be formed in the zone of antibody excess by the reaction of a second type of antibody with an antigen-antibody complex, at a different site on the antigen.

The antibody reacting in precipitation also reacts in the haemagglutination test. Sera containing precipitins usually gave high titres in the latter test (Roitt and Doniach, 1958), although occasional discrepancies were found.

Roitt (unpublished) has shown that the amount of thyroglobulin required to inhibit haemagglutination of thyroglobulin coated cells does not correlate exactly with the haemagglutination titre, and suggests that the combining power of a unit mass of antibody is variable from serum to serum.

The haemagglutinating antibody was found in the gamma-globulin fraction by electrophoretic analysis (Cline, Selenkow and Brooke, 1959; Shulman, Rose and Witebsky, 1960). Absorption of precipitins from a Hashimoto serum with thyroglobulin had no effect on the serum titre in the CF (toxic thyroid) test, indicating that antibodies are separate and distinct from one another.

The antigen in toxic thyroid glands which fixes complement with sera of persons having Hashimoto's and other thyroid diseases is distinct from thyroglobulin (Trotter, Belyavin and Waddams, 1957; Roitt and Doniach, 1958; Anderson, Goudie and Gray, 1959; Belyavin and Trotter, 1959). Sera containing complement-fixing antibody to thyroglobulin (which are uncommon) were distinguished

from sera containing antibody to the microsomal antigen and serum. A serum containing antibodies against thyroglobulin fixed complement with several dilutions of antigen, but complement was not fixed when the serum was diluted. The opposite situation, i.e. complement fixation at high serum dilutions was found with the microsomal antibody. The reactivity of a serum for thyroglobulin could be absorbed out with thyroglobulin without affecting the reactivity for the microsomal antigen. Centrifuged deposits with a high microsomal antigen content contained very little iodine and no thyroglobulin.

The antibody which fixed complement with thyroglobulin also entered into the precipitin reaction, but the antibody-antigen precipitate was capable of fixing complement. In contrast, the usual Hashimoto serum containing antibody would yield a precipitate but would not fix complement with thyroglobulin, and the antibody-antigen precipitate would not fix complement.

The antigen is present in much greater concentrations in glands from persons with Graves' disease than in normal thyroid tissue, and has also been found in increased amounts in the glands of patients with "dyshormonogenesis" (inborn deficiency of one of the enzymes necessary for production of thyroid hormone). Quantitative antigen determinations on fractions obtained by differential centrifugation showed 85% of the recoverable antigen to be in the microsome fraction (Roitt, Doniach, and Couchman; 1960). The antigenic potency of the microsomal pellet was unaffected by treatment with ribonuclease, pyrophosphate or dilute alkali. This evidence suggested that

the antigen was distinct from ribosomal RNA. The lecithinase "phospholipase C" did not change the antigenic activity of the microsomal pellet despite digestion of half of the phospholipid content. The antigen was destroyed by treatment with alcohol, acetone, butanol, deoxycholate and non-ionic detergents, trypsin and by heating to 100°C.

The variations in the antigen content of various types of thyroid gland are not due solely to cellularity of gland. Desoxyribonucleic acid determinations showed that the increase of antigen content of toxic thyroid tissue was greater than the increase of the nucleic acid content of a unit mass of tissue above the level in normal tissue. The level of antigen was found to be highest in glands of patients with most severe hyperthyroidism. Prolonged injection of thyrotrophic hormone into monkeys led to a significant fall of microsomal antigen (Roitt, Doniach, Wilson and Couchman 1960).

Holborow et al. (1960) demonstrated cytoplasmic localization of antibody by the technique of Coons and Kaplan (1950) and attributed this to the microsomal antibody, since they found it only in sera containing this complement-fixing antibody. The fixation of complement by this antigen and its corresponding antibody may be inhibited by a factor or factors present in the supernatant of thyroid extracts from which the cellular antigen has been removed by centrifugation, and in some specimens of serum and saliva (Trotter and Belyavin, 1960).

Two antigens in the colloid of human thyroid glands are demonstrable by Coons' fluorescent antibody technique. One

reacts with the precipitating and haemagglutinating antibody to thyroglobulin, and gives a characteristic pattern of fluorescence (Plate 9). Antibody to the other stains with a homogeneous fluorescence (Plate 8).

Roitt, Doniach, Wilson and Couchman (1960) separated the second colloid antigen (CA_2) from thyroglobulin by column chromatography using the method described on page 125.

The antibodies to thyroglobulin and the microsomal antigen have been shown by many workers to be in the gamma-globulin fraction of serum. Fahey and Goodman (1960) showed that the antibodies to thyroglobulin had sedimentation characteristics of both 6.6 S and 18 S gamma-globulins. The antibodies in five sera were of the 6.6 S type while both types were present in two sera.

NOTES ON THE DEFINITION OF HASHIMOTO'S DISEASE

Diagnosis of thyroid disease is unfortunately not always satisfactory; difficulty arises particularly in the region of the thyroid disease designated "thyroiditis". The difficulty in classification of thyroid disease lies in the fact that there are at least three different viewpoints from which diagnosis may be made. (Thyroid disease is not unique in this respect; however in respect to thyroid disease a particular set of clinical circumstances may have a widely variable morbid anatomical basis. According to Means (1948), "Classification based on aetiology alone is impossible, and one based on pathology or physiology alone incomplete and even misleading.")

1. Patients may be classified according to the state of function of the thyroid gland. Here there is a good agreement between the clinical state and the biochemical data. Modern techniques give a reasonably precise measurement of the metabolic activity of the gland (Hamolsky and Freedberg, 1960).

2. Clinical diagnosis. This complex process is well discussed by Wayne (1960). Conscious and unconscious reasoning is applied by every clinician, taking into account the natural history of the condition, the size and consistency of the gland, and the presence or absence of symptoms known to be associated with subnormal or excessive output of thyroid hormone. There is justifiable uncertainty in the minds of many clinicians in the diagnosis of thyroiditis. This is to some extent due to the advent of serological techniques.

3. Morbid anatomical diagnosis. The peculiar difficulty of

of the pathologist in thyroid disease contrasts with his satisfactorily authoritative position as the final arbiter in the majority of cases of human disease. Microscopically and histologically recognizable characteristics are grouped. Inferences are drawn according to general principles of pathology, but limitations are great in the study of lifeless and altered tissues as they appear in paraffin sections. Many features are common to the different groups of thyroid disease.

It is generally agreed that it is impossible to tell the functional status of the gland with any degree of certainty from its histological appearance. Hypothyroidism may be presumed where atrophy and fibrosis are extensive. There is scalloping of the edge of the colloid in paraffin sections of hyperfunctioning tissue which may be due to its resorption, the cells are numerous and tall, and they are grouped in small and infolded follicles, the opposite of their state in myxoedema. The cellularity of the gland is, however, greatly increased in cases of inborn errors of hormone synthesis (Stanbury, 1960; McGirr, 1960).

There is considerable divergence of opinion amongst pathologists in the diagnosis of thyroid disease, particularly in the group of conditions embraced by the term thyroiditis. To quote Werner (1955) in this context: "Pathologic criteria are so confused that certainty of laboratory diagnosis is impossible in many, if not most instances, and different pathologists may interpret the same section in widely varying ways. This lack of agreement makes the accuracy of statistical data derived

from different series dubious, and even within a series changing criteria require continuing classification of case material." Hazard (1955) expresses similar misgivings.

H. Hashimoto in 1912 described a histological pattern in the thyroid glands of four middle aged women who had goitres, but no striking signs or symptoms. (One patient had been husky for a month before operation, one had recently suffered from insomnia and palpitations, and one had complained of a feeling of tightness in the neck and slight earache.) Malignancy was suspected in each case and subtotal resections of the glands were carried out. The progress after operation was not reported in great detail, but convalescence was prolonged, although health was eventually regained. It is not clear whether all received thyroid preparations; in the summary Hashimoto stated: "Later, after the operation, there was a generalized œdema which disappeared after the exhibition of thyroid preparations." He concluded that "The operation causes the tumour to disappear, but one must avoid too extensive a resection. To leave part of the tumour does not do any harm. This part will, after a time, disappear spontaneously." However both lobes remained enlarged in one case.

The incidence of the condition is virtually impossible to determine. The heightened interest in Hashimoto's disease has brought about an increase in the frequency with which it is diagnosed, while past reports vary with the source of material and because of the difference of opinion of pathologists making the diagnosis. "The determination of the prevalence of struma

lymphomatosa is beset with certain difficulties of diagnostic interpretation" (Hazard, 1955).

McConahey et al. (1962) claimed that the incidence of struma lymphomatosa is increasing. In 1958 it occurred in 13% of the goitres removed at the Mayo Clinic, whereas in 1939 the incidence was less than 1%.

The condition is most easily diagnosed in women in the third and fourth decades having a firm, smooth goitre and accompanying evidence of hypothyroidism. If there is a precipitin reaction with thyroglobulin in the agar-gel diffusion test the diagnosis is certain. In the absence of the latter some cases of colloid goitre may be diagnosed as cases of Hashimoto's struma; both types of goitre may become smaller on thyroid medication.

Women are much more frequently affected than men, and the condition is most commonly seen in the latter part of the reproductive life. Children and elderly patients have been reported to be affected. The functional status of the gland, judged by clinical criteria, is most commonly normal, but it is now suspected that the common outcome, usually after many years, is myxoedema. Hyperthyroidism is sometimes seen.

Biochemical studies show a variable pattern. There is commonly a disparity between the basal metabolic rate and the uptake of radioactive iodine (I^{131}), the latter being normal or high even in the presence of clinical myxoedema and a low B.M.R. (Doniach and Hudson, 1957). I^{131} is discharged from the gland by potassium perchlorate administration (Morgans and Trotter, 1957). The protein bound and butanol extractable

radioactive iodine levels at 24 hours are usually raised, and the 48-hour plasma I^{131} activity is often high even if the patient is myxoedematous.

Thyrotrophic hormone does not increase the I^{131} uptake in Hashimoto glands, in contrast to the normal where the three- and six-hour readings are increased two- to three-fold (Levy et al., 1955; Skillern et al., 1956). Thyroxine depresses the pituitary in this condition, in contrast to thyrotoxicosis, as measured by the uptake of I^{131} (Murray and McGirr, 1960). Several groups have shown that the serum protein-bound iodine level is higher than the level of butanol-extractable iodine, which is interpreted to indicate that there is production and discharge of an abnormal iodinated protein, possibly resembling thyroglobulin.

Focal thyroiditis. Focal lymphadenoid lesions are commonly seen in sections of operation and autopsy specimens of thyroid tissue. In the great majority of cases they apparently produce no symptoms and do not constitute a clinical disease.

Focal changes resembling lymphadenoid lesions in thyroid glands of elderly women are common, and correlate well with the occurrence of complement fixing antibodies to toxic thyroid microsomes (Goudie, Anderson and Gray, 1959).

Lymphocytic thyroiditis. This type of thyroiditis is described by Hazard (1955) as a goitre containing much lymphoid tissue but without the type of epithelium found in Hashimoto's struma, and usually occurring in a young woman, an adolescent or a child. Generally there is no clinical evidence of hypothyroidism.

Gribetz et al. (1954) reported considerable differences between the butanol-extractable and protein-bound iodine levels in sera of a number of young girls whose thyroids showed this change. They suggested that the difference was due to an abnormal circulating iodinated protein.

The natural history of this group of patients has not been studied well. It may be an early form of lymphadenoid goitre.

Hashimoto's disease associated with thyrotoxicosis. Buchanan et al. (1961) compared data of 5 patients who had both thyrotoxicosis and Hashimoto's disease with 5 euthyroid cases of Hashimoto's disease who had some clinical features suggestive of thyrotoxicosis. In the euthyroid group the 24-hour uptake of radioiodine was in the thyrotoxic range, but in all of this group the PBI was well within normal limits. The 24-hour I^{131} uptake was suppressed by thyroxine therapy in all of the euthyroid patients, but not in the two cases of the hyperthyroid group in which the test was done. High I^{131} uptake in the presence of a normal PBI is therefore a valuable diagnostic lead.

The histological diagnosis of Hashimoto's thyroiditis. Whereas the clinical picture associated with Hashimoto's thyroiditis has been described in greater detail since, nothing has been added to Hashimoto's original description of the histological changes. His summary of the findings was:

(1) Rich formation of lymphoid follicles.

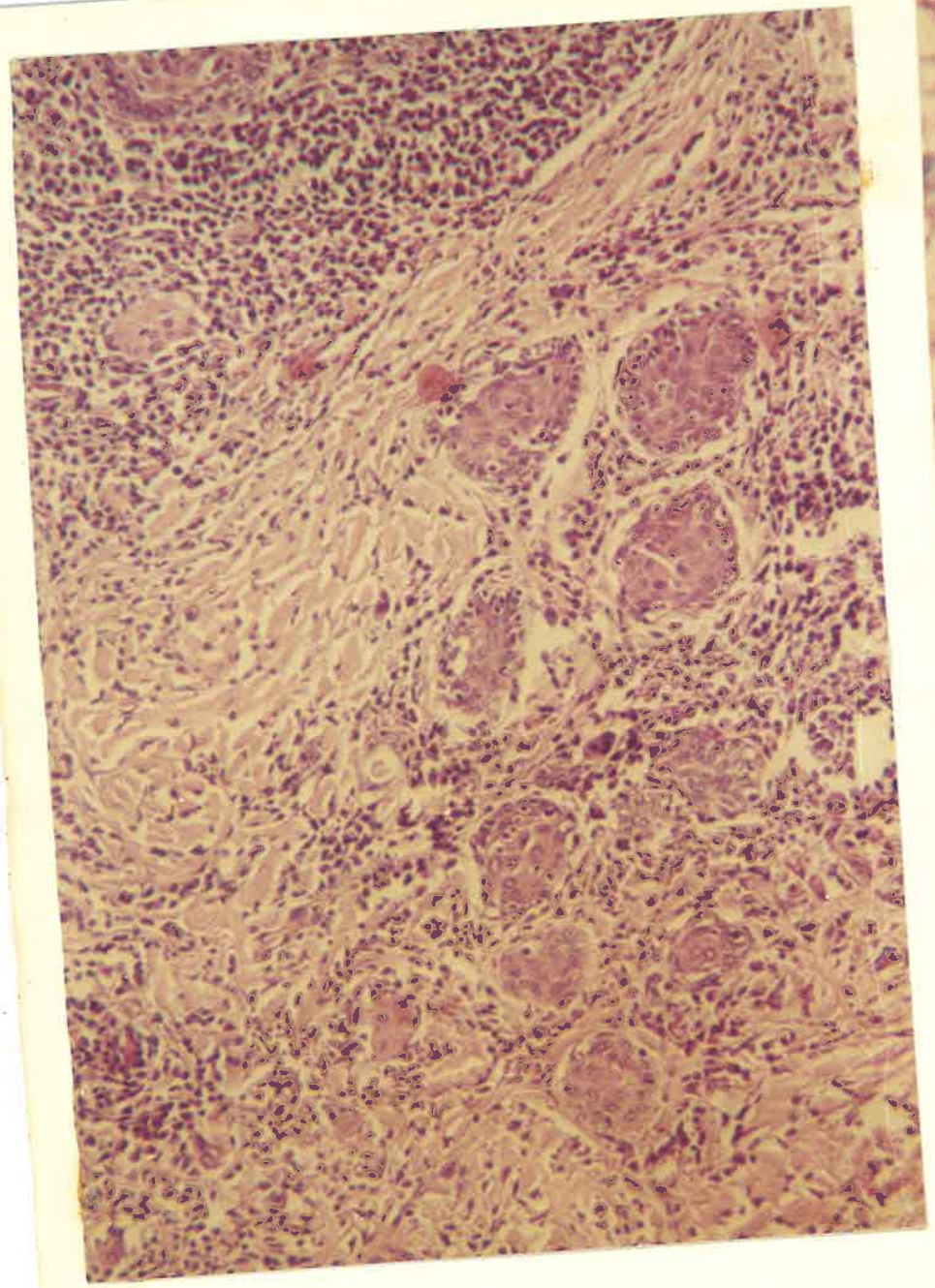
(2) A striking change in the epithelium of the vesicles and their contents.

(3) Extensive connective tissue growth.

(4) Diffuse round cell infiltration.

The epithelial change, called variously today oxyphilia, eosino-

Plates A and B. Photomicrographs of H & E section of thyroid tissue in which fibrosis is a prominent component of the Hashimoto lesion. Islands of Askanazy cells are surrounded by an inflammatory infiltrate in which plasma cells are almost as frequent as lymphocytes. Eosinophilic particles in the inflammatory exudate are probably the remains of epithelial cells.



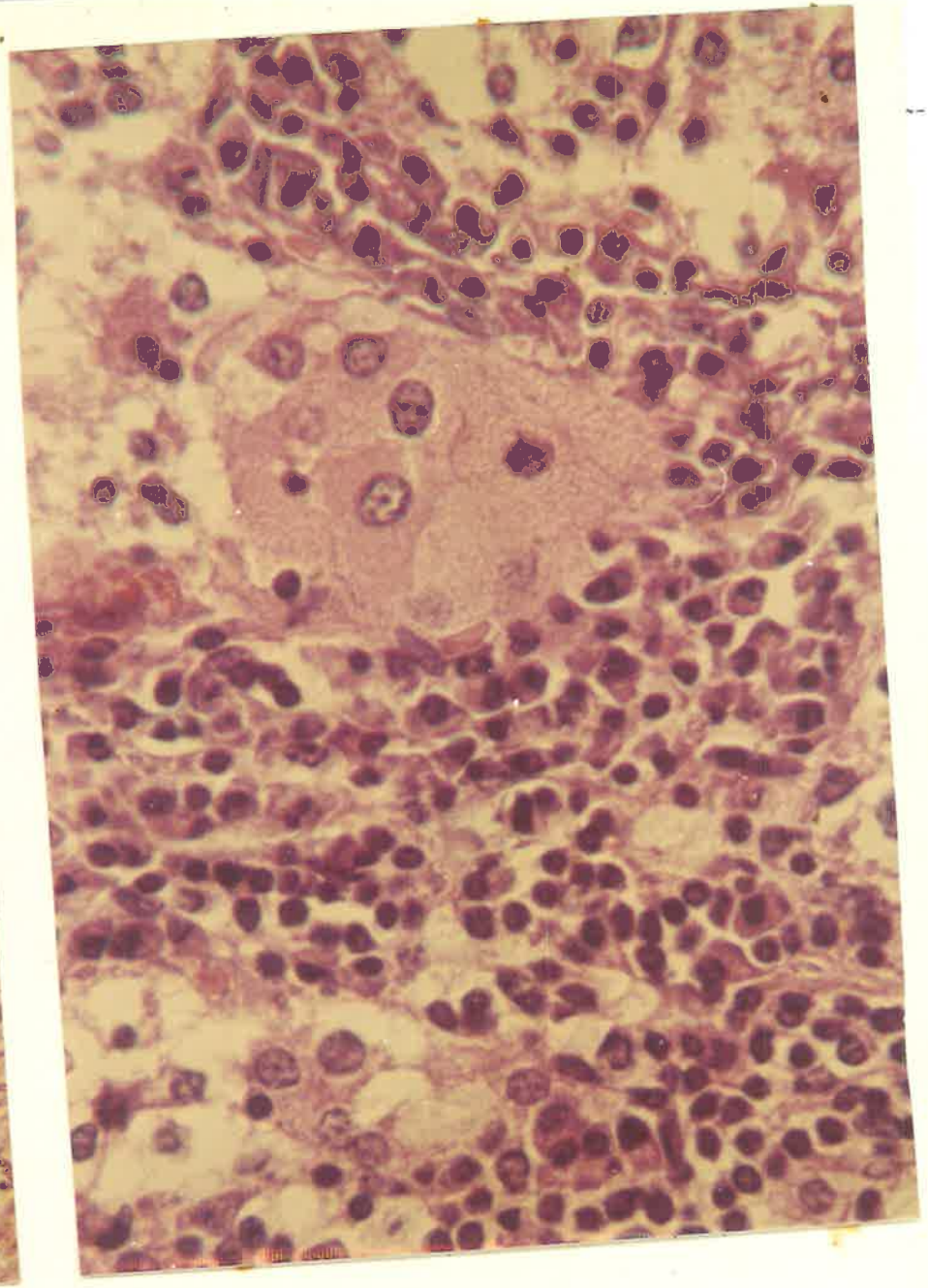
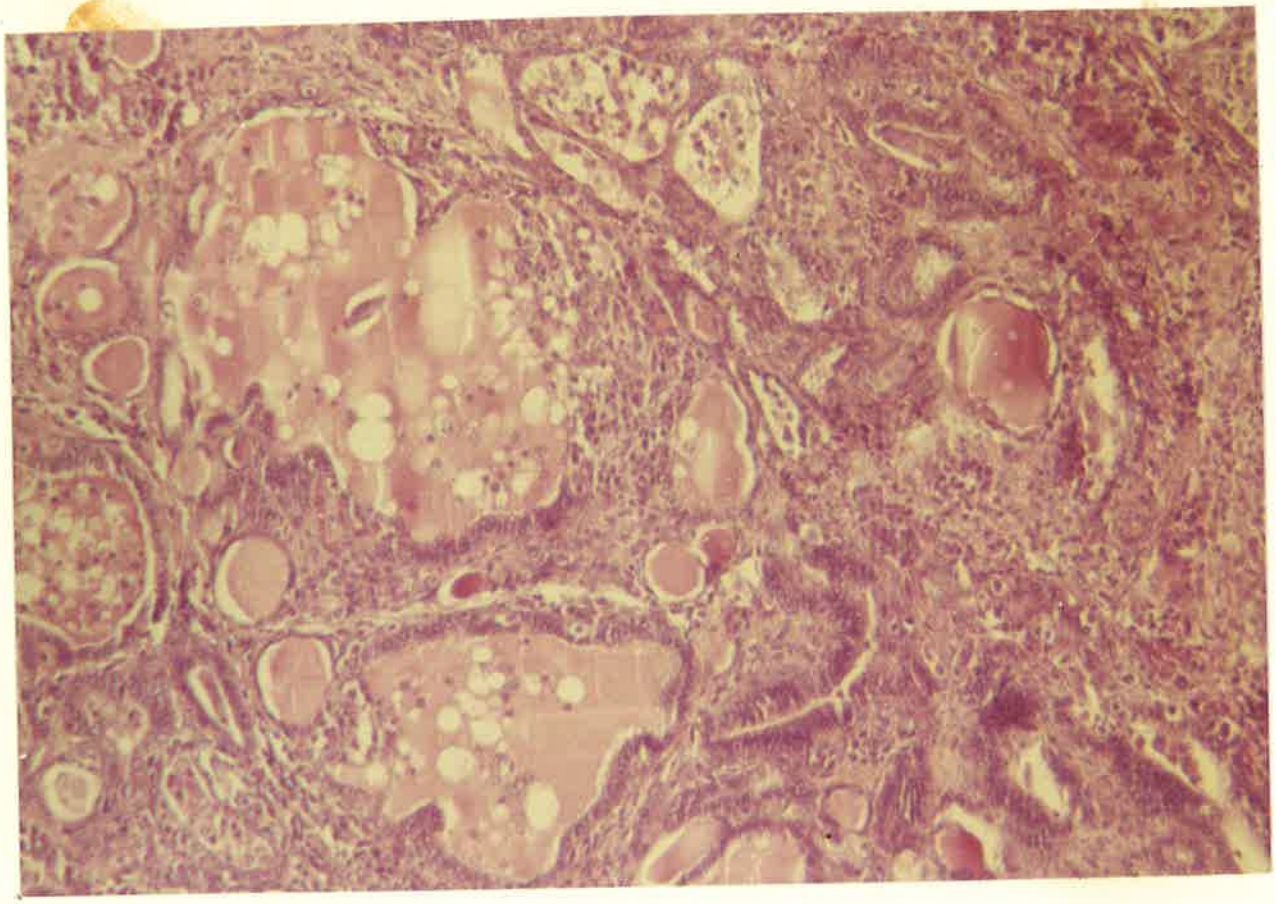


Plate C. Hashimoto's disease. In this section of the gland changes in the colloid are prominent. Vacuoles in the colloid are usually related to cells or remnants of cells. The epithelium of the largest follicle is absent over two-thirds of its circumference. Some of the cells in the colloid have the characteristics of macrophages. Others have the appearance of epithelial cells.



philia, Askanazy cell change or Hurthle cell change, was described as a granular change. The cells stained well in some areas, poorly in others and showed a variable affinity for Flemming's solution, Sudan III or Scharlach red. Lymphocytes were seen in the cell body and between the cells. Changes in the colloid were also described - degeneration and the presence of epithelial cells and macrophages.

It would seem best to broaden the histological criteria for the diagnosis of Hashimoto's disease somewhat. If it is accepted that lymphocytic and plasma cell infiltration, epithelial hyperplasia and oxyphilia, and fibrosis are the main histological manifestations of the condition, it is not unreasonable to expect the relative prominence of each of these features to vary from case to case. Hashimoto indicated as much himself.

Since the relationship of focal to diffuse thyroiditis is obscure, a distinction should be made between the two conditions, even though the ingredients of the latter are present in focal lesions. Woolner, McConahey and Beahrs (1959) classified the pathological status of glands of 605 cases of "thyroiditis of the Hashimoto type" seen during a 27-year period at the Mayo Clinic (see Table 1).

Table 1

WOOLNER, McCONAHEY AND BEAHRS (1959)

CLASSIFICATION OF HASHIMOTO'S THYROIDITIS.

	Number of cases
Diffuse thyroiditis	
Pronounced epithelial destruction	29
Oxyphilic epithelium	36

Table 1
(continued)

	Number of cases
Varied epithelial changes (not predominantly oxyphilic)	106
Lymphoid type (including lymphosarcoma)	17
Thyroiditis with "hyperplastic" epithelium	57
Focal thyroiditis	360

They accepted variations within a theme of Hashimoto's original description: for example, a large proportion of cases in which there was dense lymphocytic and plasma cell infiltration had minimal hyperplastic or oxyphilic change of the thyroid epithelium. They were cautious, however, in drawing conclusions as to the course of the disease as consecutive histological material is not frequently available. The frequent occurrence of myxoedema suggests a progression, but they quoted a case of a woman who had moderate thyroiditis with numerous lymph follicles at the age of 22 for which a subtotal thyroidectomy was performed. Recurrent goitre was similarly removed 6 years later, and there was no apparent progression of the histological lesions. At the age of 48, 50 g. more tissue was removed, still showing spotty thyroiditis scarcely, if at all, advanced in severity. She was euthyroid, and had a soft recurrent goitre at the age of 58.

Myxoedema was the usual outcome after biopsy of either a minor or major portion of a gland diffusely involved by thyroiditis. Recurrence of goitre after surgical removal was common, but where there was extensive epithelial destruction goitre did not recur. Cases having hyperplastic epithelium were more common in the younger age groups. Cases with extensive epithelial destruction frequently had advanced or advancing fibrosis.

These authors summed up their impressions in this way. "It would appear that Hashimoto's thyroiditis is characterized mainly, in its advanced form, by diffuse interfollicular infiltration with plasma cells and lymphocytes. The associated epithelial changes suggest varying degrees of follicular damage. Spotty or even total destruction of epithelium is present in some cases." They felt that there was not sufficient difference between the clinical and pathological findings in the four subgroups of diffuse thyroiditis to warrant regarding any one of them as the true syndrome. There is insufficient evidence, they stated, to be sure of the relationship of focal to diffuse thyroiditis, and to know whether the condition is frequently progressive.

Pain, Terplan, Rose, Witebsky and Egan (1957) have different criteria for the diagnosis of Hashimoto's disease, namely:

(1) Diffuse dissemination of lymphoid tissue, with lymph follicle formation.

(2) Parenchymal degeneration.

(3) Absence of non-lymphocytic infiltration (polymorphonuclear leukocytes, plasma cells or giant cells).

Askanazy cell change was not mentioned by these authors. They stated that they were reluctant to use the specific histologic term of struma lymphomatosa unless a uniform lymphadenoid transformation of each lobule was present. This group used the term "chronic non-specific thyroiditis" to include glands presenting a varied picture of histological changes differing from Hashimoto's disease, Riedel's struma and from the de Quervain type of thyroiditis. It included cases in which large numbers of plasma cells were seen, which they would otherwise have included in the Hashimoto group. They wrote: "A more specific classification of these cases is impossible unless the criteria of the original descriptions of de Quervain, Hashimoto and Riedel are to be so broadened that the original concepts lose their significance." The difference in criteria of diagnosis is reflected in the results of antibody studies by Paine et al. which differ considerably from the results of other workers.

From these notes, which are intended to introduce briefly the subject of autoimmunity in thyroid disease, it should be clear that the definition of thyroiditis is not much more precise than it was after Hashimoto published his paper in 1912. No unique biochemical changes have been discovered. It is by no means certain that de Quervain's thyroiditis is always an entity distinct from Hashimoto's disease.

Roitt and Doniach have used the term "auto-immune thyroiditis" in their writings, although this term has not been clearly defined. They use the term loosely, with justification, to include all types of thyroid disease in which thyroid auto-antibodies are found frequently and in high titres (Table 2). It

Table 2

ROITT, HUDSON AND DONIACH'S CLASSIFICATION OF THYROIDITIS (1961)

I Infectious thyroiditis

A. Bacterial - Acute: Suppurative; Non-suppurative

Chronic: Non-specific (pyogenic); Specific (tuberculosis, actinomycosis, syphilis)

B. Viral - Subacute giant cell thyroiditis of de Quervain.

Chronic varieties of de Quervain (granulomatous pseudo-tuberculous thyroiditis)

II Radiation thyroiditis

III Auto-immune thyroiditis

A. Diffuse (struma lymphomatosa)

(1) Classical Hashimoto's disease.

(2) Lymphocytic thyroiditis.

(3) Atypical forms of lymphadenoid goitre.

Hashimoto's associated with thyrotoxicosis.

Auto-immune thyroiditis with subacute onset.

Remittent auto-immune thyroiditis (with silent episodes).

Hashimoto goitre with marked collagen proliferation.

(4) Primary myxoedema (atrophic thyroiditis).

B. Focal - in "normal glands" and in various underlying goitres.

IV Riedel's thyroiditis (struma fibrosa with invasion of surrounding neck structures).

V General pseudo granulomatous diseases invading the thyroid: sarcoidosis and amyloid disease.

is implied that diffuse or focal thyroiditis is the underlying lesion from which the auto-antibodies arise. In order to gain evidence for or against this concept a correlation of histological appearances with serological findings was carried out by Dr. D.A. Senhauser of the Cleveland Clinic, Cleveland, Ohio, U.S.A. Preliminary impressions have been presented (Doniach, Roitt, Forbes and Senhauser, 1961) and will be described briefly.

Material from 75 glands was studied. The four main histological groups were:

(1) Struma lymphomatosa, oxyphilic variant (classical Hashimoto's disease). 27 cases.

(2) Struma lymphomatosa, fibrous variant. 30 cases.

(3) Lymphocytic thyroiditis. 2 cases.

(4) Multifocal thyroiditis. 7 cases.

Nine cases showed a picture intermediate between the oxyphilic and fibrous variants.

The glands included in the first group showed large polyhedral cells with diffuse oxyphilia (Askanazy or Hurthle-cell change). The acini were uniformly small, containing small amounts of deeply staining colloid, or were devoid of colloid. The lobular architecture of the glands was largely intact. Scattered throughout the sections were varying numbers of lymphoid follicles with prominent germinal centres. Extending from these follicles into the interstitium there were inflammatory cells composed primarily of lymphocytes with scattered plasma cells. Serologically this group of patients was characterised by overall lower antibody titres though all without

exception had circulating antibodies to at least one of the three known thyroid auto-antigens. Only 2 patients had precipitins and 2 more had TRC titres of over 250. C.F.T. was positive in 23 cases and in approximately half of these the titre was 1/64 or over. The fluorescent test for CA2 antibodies was positive in practically all cases.

In the 30 cases belonging to the fibrous variant, the glands showed varying degrees of architectural disruption with replacement fibrosis. In most instances areas of active fibroplasia were seen although several cases suggested a "burnt out" condition with dense hyaline fibrosis in which small nodules of oxyphilic epithelium remained. In the majority of these glands oxyphilic change was present to a varying degree. The round-cell infiltrate varied markedly in its intensity but in most composed predominantly of plasma cells. Lymphoid follicles were present but in lesser numbers than in the oxyphilic variant. In advanced lesions there were few remaining acini, and many of the epithelial cells appeared in solid nests some of which showed squamous metaplasia. Serologically these patients had precipitins in every instance with TRC titres of over 1/25,000 in all cases. C.F.T. titres were also higher in general with 2/3 of patients having titres of 1/64 to over 1/512. The incidence of CA2 antibodies could not be determined owing to the presence of high titre thyroglobulin antibodies.

The third group was much smaller owing to the surgical origin of the material. The 2 cases of lymphocytic thyroiditis were characterised by absence of oxyphilia and a less marked

lymphoid invasion. The prominent feature was a marked colloidophagy with numerous macrophages in the thyroid acini and multifocal areas of epithelial hyperplasia in spite of the euthyroid state of the patients. The thyroid was diffusely affected in these cases but the degree of thyroiditis varied somewhat in different parts of the gland.

In the 7 cases of multifocal thyroiditis, a category which is usually considered separate from lymphadenoid goitre, the same lesions were seen but with areas of histologically intact thyroid tissue between lesions. In the areas of thyroiditis there were a few oxyphilic epithelial cells and colloidophagy with lymphoid follicles and scattered lymphocytes. Serologically these patients had low titre antibodies, none had precipitins and C.F.T. titres were usually under 1/64. The fluorescent test for CA2 antibodies was positive in all the cases.

There were 9 further cases of struma lymphomatosa which appeared to be intermediate between the oxyphilic and fibrous groups in that histological features of both variants were present.

Four cases appeared to be progressing from the oxyphilic lesion to an unusually intense active fibroplasia and the remaining 5 appeared to have lymphocytic thyroiditis since they showed little or no oxyphilia, yet they had an appreciable amount of fibrosis and were progressing to the fibrous variant. Serologically these intermediate cases all had high antibody titres with precipitins and a high C.F.T.

In conclusion it would seem that the more intense the destruction and fibrous replacement in the thyroid gland, the

higher the amount of circulating antibodies produced. This is also reflected by the predominence of plasma cells in the fibrous glands. The oxyphilic change may well prove to be connected with "cell-bound" antibodies or other immunological mechanism dependent upon lymphocytes whereas the circulating antibodies are more likely to represent a secondary reaction due to antigens released from disrupted acini. This study further suggests that the fibrous group of lymphadenoid goitres is composed of two types of cases. Some patients especially males, have a rapidly developing large goitre with massive active fibrosis whereas others with smaller goitres and a more prolonged course may be the end result of the oxyphilic variant in the majority of cases, or else derive directly from the lymphocytic thyroiditis without going through the oxyphilic stage.

Schade, Owen, Smart and Hall (1960) correlated the histological findings with the occurrence of thyroglobulin and microsomal complement-fixing antibodies. A highly significant association was found between the presence of antibodies (either or both) and lymphocytic infiltration. Microsomal antibody contributed little to the correlation, although the presence of this antibody was significantly related to the diagnosis of Graves' disease.

Schade et al. considered their findings evidence of local formation of thyroglobulin antibody, but felt that the site and manner of formation of microsomal antibody was uncertain. They suggested that microsomal antibodies may belong to a "different order of immune defense" adapted to function in the circulation rather than in the tissues.

Auto-immunity - the presence of circulating auto-antibodies - is not an accompaniment of all cases of thyrotoxicosis or myxoedema, but if all known types of thyroid antibodies are tested for, cases of histologically proven Hashimoto's disease without auto-antibodies are rare. (No such cases were found in the series reported in this thesis.) Auto-immunity may well be the indicator of a biochemical lesion which is common to various clinical types of thyroid disease. This lesion may occur only in those hereditarily predisposed (Hall, Owen and Smart, 1960; Table 26).

STUDIES WITH THYROID TISSUE CULTURES ON THE ACTION OF HUMORAL FACTORS IN THYROID DISEASE

The problems of auto-immunity must be studied in living cell systems. The eventual proof that auto-immunity is responsible for human disease must be obtained by demonstration of metabolic effects caused by antibodies or immunologically competent cells in situations as closely resembling the naturally occurring state as can be devised.

The serological approach is valuable to indicate diseases in which autoimmunological activity occurs, but the intimate effects of auto-antibodies or cells bearing antibodies must be studied at a cellular or sub-cellular level.

Thyroid tissue culture was undertaken as a step towards this goal. The action of serum factors on living cells can be studied in this way, and although the cells are in an environment far removed from normal, and are themselves certainly altered as a result, useful information has been obtained with this tool.

METHODS

Tissue Culture: The materials used are described in Appendix 1. Thyroid tissue was obtained fresh from the operating theatre. It was placed in a polythene bag for transport. Before the culture procedure was started, the gland was examined by a pathologist who took material for histological section. This unavoidable break in the aseptic routine had no apparent harmful effects. Generalized infection of the cultures did not occur, as would be expected if the cell inoculum were seriously contaminated. This was due to three factors; the primary digest of cells was discarded, the culture medium contained antibiotic, and the cultures were kept for only a day or so.

The technique for trypsin-dispersion of thyroid cells was as follows. Approximately 5-10 g. of gland were minced in a petri dish containing phosphate-buffered saline (PBS Dulbecco) by cutting with two scalpels moving in opposite directions. The buffer was decanted, and the tissue put into a stoppered flask containing 50 ml. of 0.25% trypsin in PBS. This was incubated in a water bath at 37° C. for 15 minutes, then the supernatant was discarded. 100 ml. of fresh trypsin solution was added and the tissue was incubated for a further hour. The bottle was shaken four or five times during this process. The supernatant was then decanted through sterile gauze and centrifuged at approximately 600 r.p.m. for 5 minutes. (Slow centrifugation is necessary to avoid cell damage and to achieve some degree of differential centrifugation between thyroid cells and erythrocytes. This was found by trial and error.) The cells were resuspended once in approximately 30 ml. of PBS and re-centrifuged. The centrifugate of washed

cells was resuspended in 2-3 ml. of Parker 199 medium.

The cells at this stage were present individually and in small and large aggregates of polyhedral cells, retaining the relationship they had to each other in the intact acinus. The larger clumps were often saucer-shaped, evidently representing segments of the follicular walls. They may contain a hundred or more cells.

The correct cell density was judged by examining a drop of the final suspension on a slide by phase-contrast microscopy. Not more than five clumps of cells should be seen in one field using a 10 x objective. This density was achieved by adding the concentrated suspension drop by drop to the required volume of Parker 199 (0.6 ml. per chamber).

This method of judging the cell density of the inoculum was used as it was impossible to count the thyroid cells. The thyroid cells grow mainly from clumps, the individual cells apparently not surviving well. A haematocrit examination would not give an estimate of the number of clumps.

If the cell concentration was too high the end-point of the test was not clear. A strongly cytotoxic serum killed all cells in an inoculum 24 times stronger than one prepared as described, but a weakly cytotoxic serum allowed a proportion of cells to survive, and the proportion of surviving cells increased greatly in increasingly dense inocula, as all of the cytotoxic factor was absorbed.

The cell suspension was mixed with serum to be tested in the desired proportions, and pipetted into the chambers, which were then sealed with a coverslip, and placed coverslip-down-

wards in a suitable tray for incubation at 37° C.

The chambers were usually examined with a phase-contrast microscope after incubation for 18 hours.

Preparation for testing sera for cytotoxic factor. Test sera were measured into bacteriological tubes (3" x ½") in racks, this usually being done on the days preceding an experiment, and the sera were stored at -20° C. until shortly before use. Complementing serum was added on the day of the experiment before the addition of the cell inoculum. This latter step was done in small batches to avoid loss of cells through adhesion to the glass of the test tube. The contents of the tube were then pipetted into the chambers, which were sealed with a coverglass and labelled. Adhesive labels were written out in advance to speed the operation.

Concentration of reactants in the system. Early experiments were performed using a 50% concentration of test serum, the other half of the medium being Parker 199 containing trypsinized cells in suspension. Subsequent experiments showed that there was no detectable difference in the vigour of the growth of thyroid cells in serum concentrations varying from 10% to 50%. Early investigations showed that a heat labile factor, presumably complement, was necessary for the demonstration of cytotoxicity. Other experiments showed that in order to be sure of the presence of an adequate amount of complement, an equal volume of fresh normal human serum to that of the test serum was necessary.

The proportions of reactants used routinely were:-

Cell suspension in Parker 199 medium	0.6 ml. (75%)
Test serum	0.1 ml. (12½%)
Fresh normal human serum ...	0.1 ml. (12½%)

Various attempts were made to avoid the use of fresh normal human serum for complement. The human serum must be obtained from previously tested donors, to avoid the risk of contaminating all chambers with cytotoxic factor. Guinea-pig serum preserved by Richardson's method, either in solution or as a freeze-dried powder (Burroughs Wellcome Ltd.) contains sodium azide and was therefore unsatisfactory. Fresh guinea-pig serum is satisfactory in most cases, but occasional sera are potently toxic; if it is to be used it is desirable to test it in advance, and to store the serum at -20°C. Fresh normal human serum from a small group of donors was used in most of the work to be described.

Precautions and controls. The system must be controlled by showing satisfactory establishment of cells in a normal serum environment, and by demonstrating that the cells are sensitive to the cytotoxic factor.

Five known cytotoxic sera were set up routinely. Some of these were potently cytotoxic and one or two were weak. Test sera were stored in small amounts to avoid loss of potency which occurs with repeated freezing and thawing. All sera were stored at -20° C

Two known non-toxic sera were set up routinely to show that there was satisfactory growth of the cells used in the absence of the cytotoxic factor. One of these test sera was

that added to all test chambers in order to supply complement.

Initial experiments were undertaken to establish that the numerous possibilities for error in setting up cultures were eliminated. It is necessary when starting up tissue culture work to be sure that the technique for cleaning of glassware is satisfactory in that no nonspecific cytotoxic substances are contaminating vessels and chambers. The distilled water supply may not be satisfactory. Certain types of rubber in tubing and bottle caps may be injurious to cells. (All screw cap bottles were treated with silicone, and the rubber caps were replaced by a synthetic elastomer, which can withstand 160° C. "silastomer 55T", Richard Klinger, Sidcup, Kent.)

Sera were stored in Wasserman tubes, stoppered with polythene bungs.

Case material. Sera were obtained from patients at the Middlesex Hospital who were attending the Thyroid Clinic or outpatient clinics, and from some patients in the wards. Patients attending the Thyroid Clinic were seen by Dr. D. Doniach, who also saw most of the other cases from the Middlesex Hospital. Some sera were referred from other hospitals in England for thyroid antibody tests. In these cases a questionnaire was sent back to the practitioner to obtain clinical and laboratory data.

Data were recorded on case cards in the laboratory from information in the Middlesex Hospital case records and from the questionnaires.

Copies of all histological sections were kept in the laboratory. Needle biopsy was carried out on many cases from

the Middlesex Hospital by Dr. R. Turner-Warwick. Copies of sections were obtained from other hospitals.

Whenever it was possible the relatives of 54 propositi with thyroid disease were seen at the hospital by Dr. Doniach or myself, and were questioned and examined for evidence of thyroid disease.

Cases of primary myxoedema and thyrotoxicosis were accepted if the clinical evidence was unequivocal or if confirmed by laboratory studies. Cases of non-toxic nodular goitre may have included some in which focal thyroiditis was present but was not disclosed by laboratory studies. The cases of Hashimoto's disease were accepted on the criteria of myxoedema with a firm smooth goitre which became smaller on thyroid therapy, or in euthyroid patients with diffuse goitre who had confirmatory evidence from I^{131} studies and serum protein abnormalities. The diagnosis of Hashimoto's disease was proved histologically in 31 of the 53 cases tested; of the remainder, 20 gave a positive precipitin test. Sera were obtained from 118 relatives (72 siblings, 30 offspring and 16 parents) of 54 propositi with thyroid disease, 46 with Hashimoto's disease, 2 with biopsy-proved focal thyroiditis, 4 with thyrotoxicosis, and 2 cases of suspected Hashimoto's disease.

Ninety-one control sera from persons without overt thyroid disease were obtained from the antenatal clinic (21 sera) and from the routine pathology laboratory. Diagnoses were obtained from information in the case notes. Sera of 27 blood donors, 8 healthy young adult medical workers, 4 relatives of an 11 year old girl with systemic lupus erythematosus and 31 hospital patients were also tested. The diagnoses of the latter were:

orthopaedic conditions (6 cases), gingivitis, hepatic cirrhosis with aplastic anaemia, dysmenorrhoea, migraine, allergic rhinitis, ulcerative colitis, Addison's disease, parotitis, idiopathic thrombocytopenic purpura, anxiety states (3 cases), hypopituitarism, obesity (2 cases), lupus erythematosus (5 cases), diabetes mellitus, rheumatoid arthritis, ischaemic heart disease, hypotension of unknown aetiology, idiopathic haemolytic anaemia.

Tests for thyroid antibodies. Coons' fluorescent antibody technique (Plates 8,9,10) was used for the determination of cytoplasmic staining of unfixed frozen sections of toxic thyroid gland as described by Holborow et al. (1959). Antibodies to the colloid were demonstrated by the fluorescent antibody technique using alcohol-fixed thyroid sections (Balfour et al., 1961). The tanned-cell haemagglutination test for thyroglobulin antibodies was carried out with a formalized sheep cell preparation (Fulthorpe et al., 1962). The complement fixation test was done with toxic thyroid gland homogenate using 2 minimal haemolytic doses (MHD) or 1½ MHD of complement (Roitt and Doniach, 1958). The precipitin test (by the Ouchterlony (1948) method) was not done when the thyroglobulin haemagglutination test was negative. A negative result is scored either when the test was negative in the presence of haemagglutinating antibodies, or when haemagglutinating antibodies were absent and there was no precipitin pattern in the fluorescent antibody test for localization of gamma globulin to the colloid. Coons' fluorescent antibody technique as applied to the colloid was recorded as negative, or positive with a homogeneous fluorescence (+S) or a precipitin pattern (+P). (A homogeneous pat-

tern in the presence of a low titre of thyroglobulin antibody may be due to localization of antibody to thyroglobulin or to the second colloid antigen.)

THYROID CULTURE: MORPHOLOGY

Trypsinization causes the liberation of cells singly and in plates from the follicular wall. When a suitable concentration of these aggregates is inoculated into a medium containing growth requirements and serum, the clumps of cells begin to adhere to glass within 30 minutes and reorientate themselves by extending pseudo-podial processes and moving away from the main cell mass. Within 18 hours in favourable conditions most of the cells are spread on to the glass. Healthy cells form flat sheets or a reticular pattern with cells connected by short processes. Some cells are connected to others by extremely long thin processes. The cells are usually bipolar, but become increasingly flatter with age. The cytoplasm contains filamentous mitochondria which are difficult to resolve, and a variable number of dark spherical granules of unknown nature. These increase in numbers in old cultures, particularly if infection is present or if the medium is not renewed.

The nucleus is usually translucent except for a few threads and clumps of nuclear material. The nuclear membrane is a thin structure. Cells are often binucleate.

Lymphocytes are commonly present although the numbers vary greatly with different glands. They are most numerous in glands which are affected by Hashimoto's disease.

Thyroid cells in culture rarely undergo mitosis; in fact this was never seen in 49 cultures, although Irvine (1960) and Pulvertaft (personal communication) have each had a culture in which mitoses occurred.

Plate 1. Phase-contrast-photomicrograph of a large aggregate of thyroid cells released by trypsin-digestion of thyroid tissue obtained from a patient with Graves' disease. The cells were inoculated into the culture chamber and had been incubated for 30 minutes. The darker central area of the clump is comprised of cells which have already become adherent to glass.

Plate 2. Cells from the inoculum shown in Plate 1 , 2 hours later. All of the cells on the surface of the clump adjacent to the glass have become adherent to it, and some cells have migrated from the aggregate. Two migrating cells are seen well in the lower right margin of the clump.

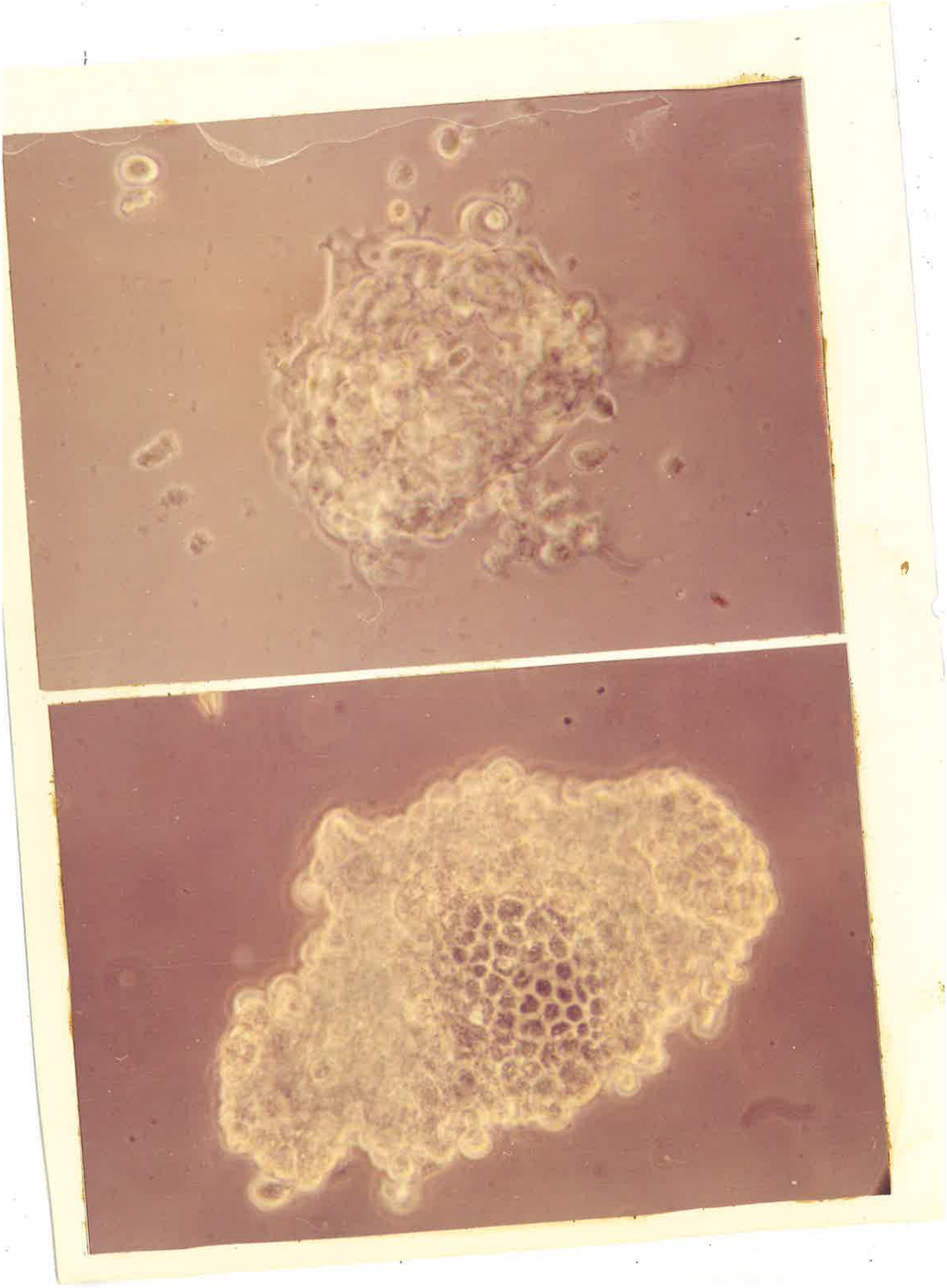
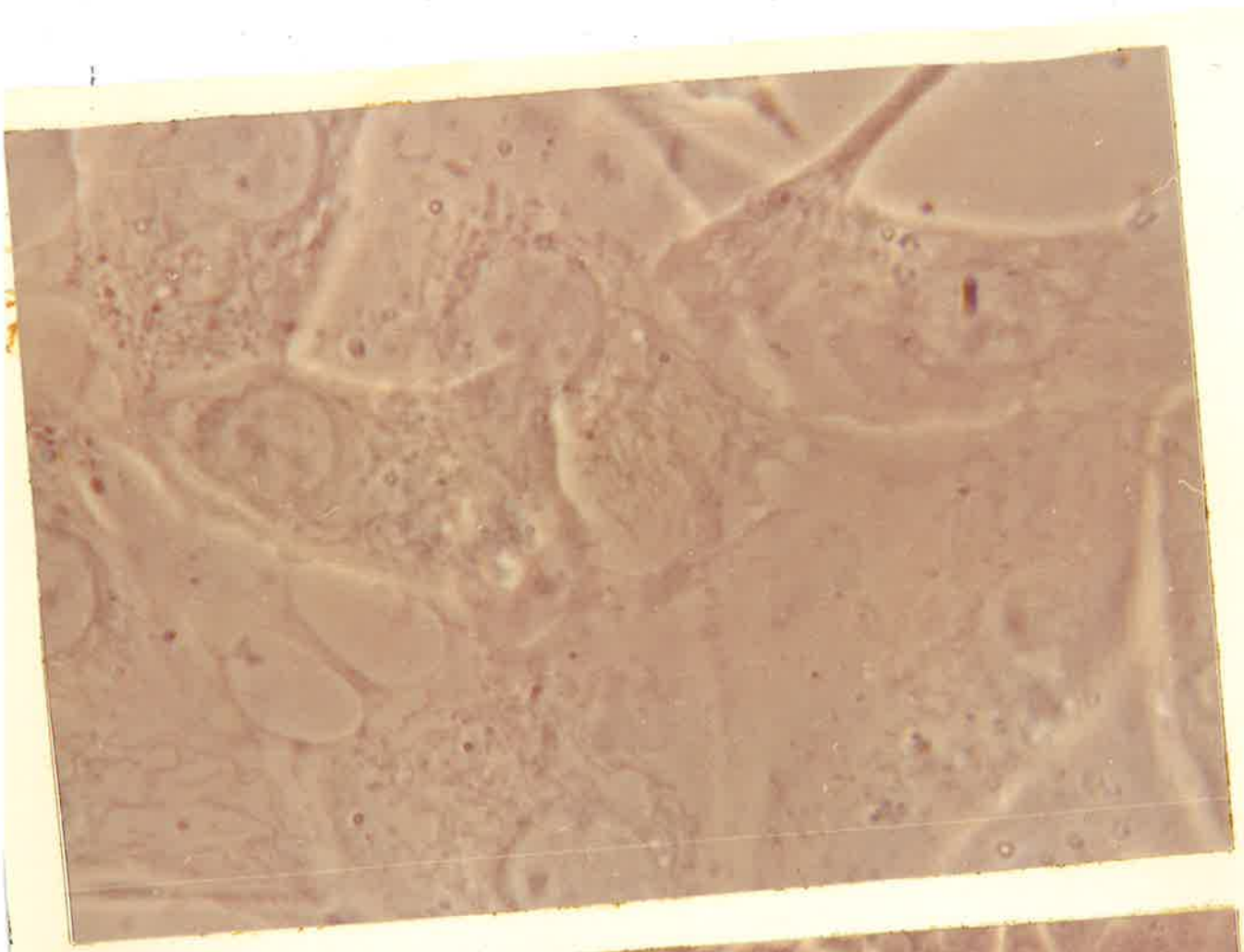
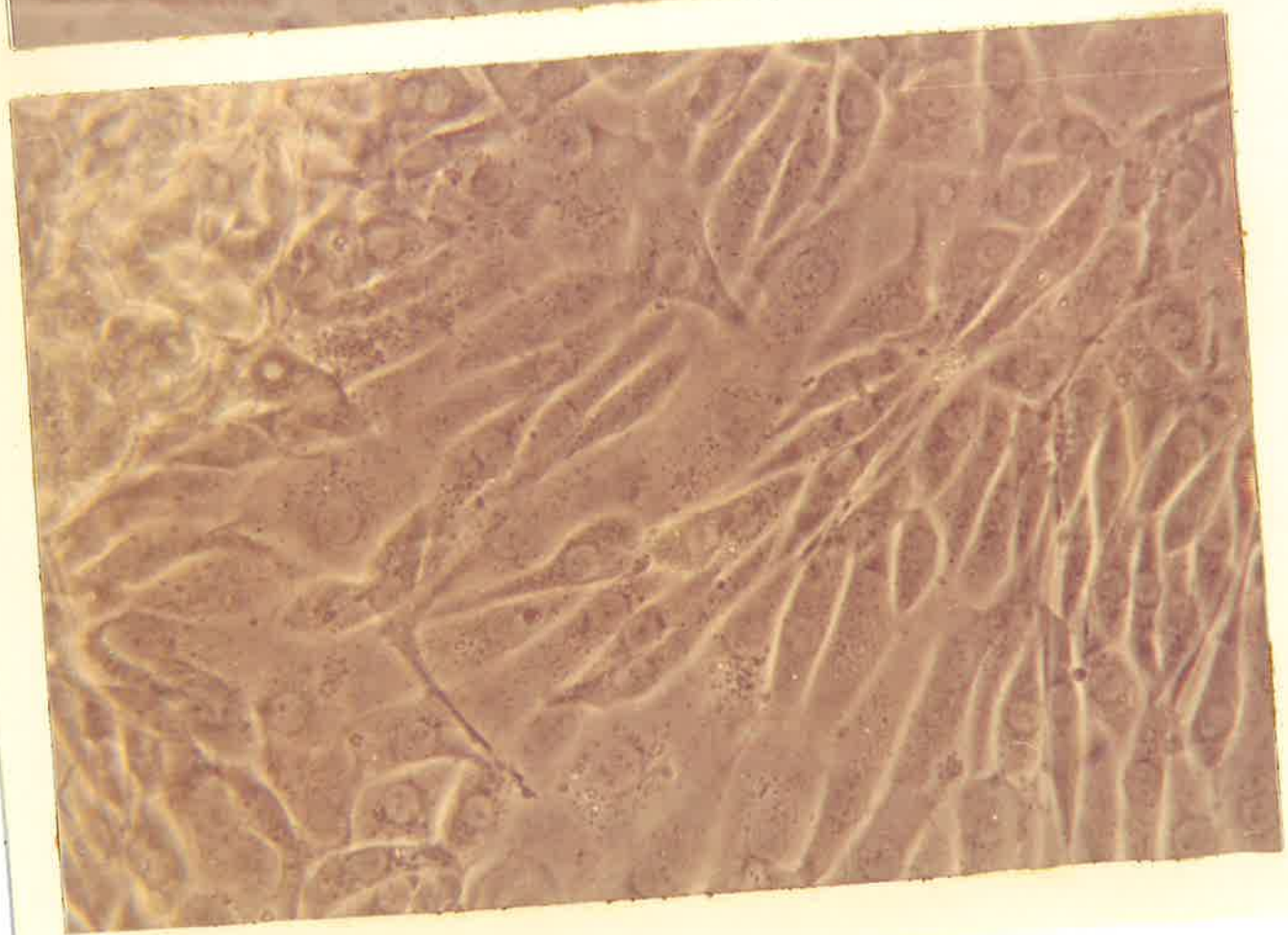


Plate 3. Exuberant "growth" of toxic thyroid cells in a heavily inoculated chamber at 24 hours. A dense clump of cells in the bottom right hand corner has not flattened out over glass; in a lighter inoculum clumps have usually become spread at this stage. In this dense field some cells can be seen overlying others. Irvine (1960) thought this to be a characteristic of thyroid cancer cells. It only occurs in dense cultures of non-malignant cells.

Plate 4. Phase-contrast micrograph of healthy toxic thyroid cells at 24 hours (oil immersion photograph of the culture shown above). Cells tend to be bipolar early when crowded, but become broader and more thinly spread when they have plenty of room, after a day or so. Note particularly the long filamentous mitochondria. These can be resolved adequately with critical optical conditions, although they are not dense. The nuclei are translucent. One or two irregular masses of chromatin are seen. These become more distinct with the passage of time. The nuclear membrane is a fine structure. Occasional dark spherical cytoplasmic granules are seen.





It is convenient to speak of "growth" of thyroid cells in culture, although the cells neither multiply nor increase perceptibly in mass.

If nourished adequately thyroid cultures may survive for 8 weeks. Pulvertaft et al. (1959) described a multicentric transformation occurring in old cells to a type with characteristics of neoplastic tissue in vitro. Transformed cells are spindle shaped, irregular in size and arrangement, and mitoses are seen.

The effects of the cytotoxic factor: determination of the end-point of the test.

The effect of Hashimoto disease serum is shown on human thyroid cells in primary tissue culture, in either of two ways.

When thyroid tissue is cultured in the presence of a cytotoxic serum, and the culture is examined at approximately 18 hours after incubation a most decisive result is obtained.

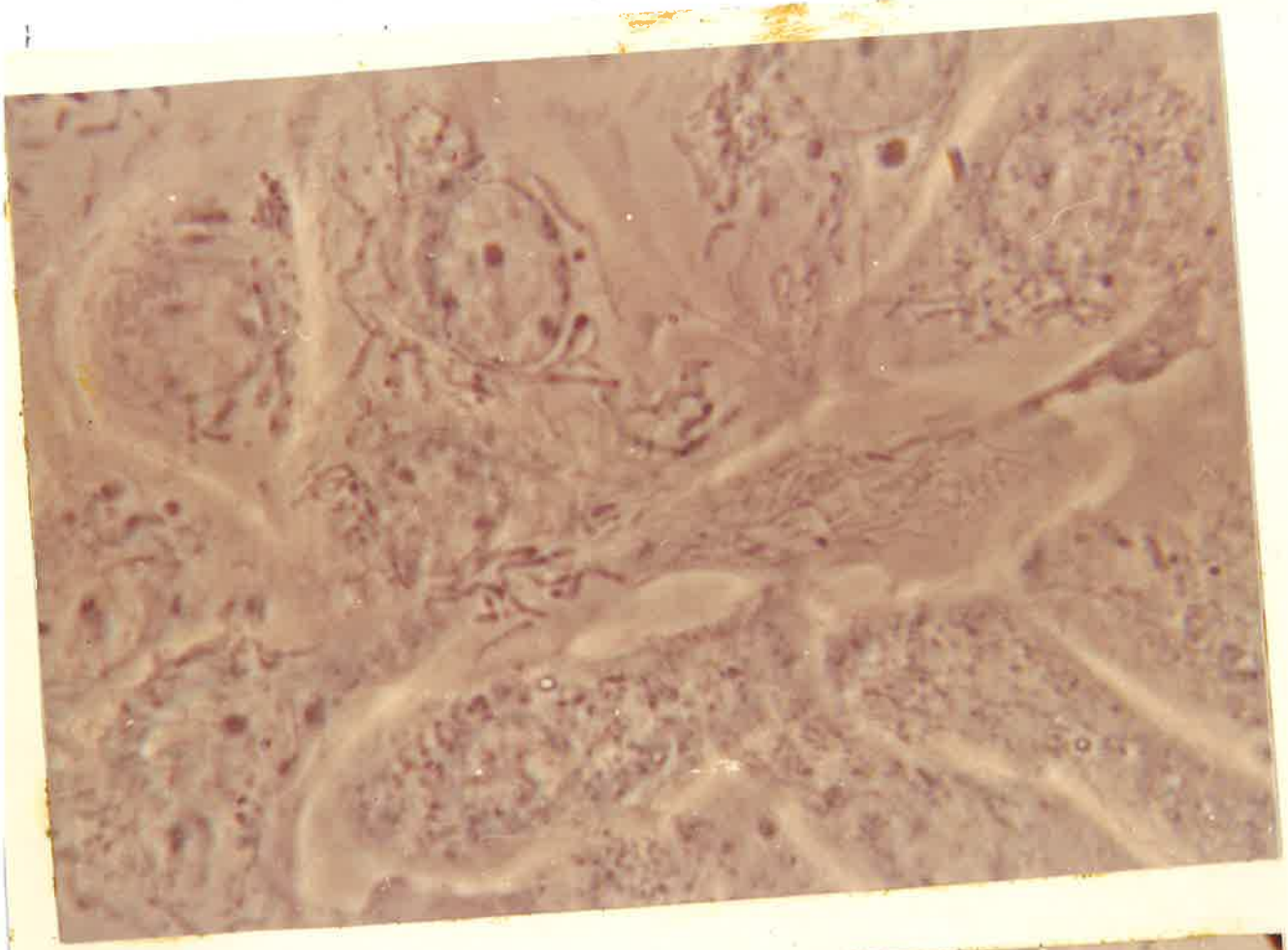
The cell clumps are degenerate. There is no extension of pseudopodial cytoplasmic processes, the first stage in centrifugal migration of the cells to form a monolayer. The cells are granular, and a proportion of them, which increases rapidly with the passage of the hours, is disintegrating. Dead but unbroken cells have different refractile properties, appearing dull grey, and lacking a glistening quality, particularly of the cell border, which is possessed by living cells. A faint fluid filled balloon can often be seen, the distended cell membrane which has lost its osmoregulatory capacity. Coagulated cytoplasm surrounds the nucleus.

Secondly, by adding Hashimoto's disease serum to a freshly established culture, at about 18 hours, cytotoxic effects can be observed if the culture is studied by phase contrast microscopy at 37° C. This is best seen by time lapse cinematography.

The morphological changes are as follows: Within 30 seconds the normally thin and pale filamentous mitochondria darken, retract and thicken. They become spherical, enlarge and become hollow vesicles. About 5 minutes after the changes in the mitochondria, the cytoplasm retracts, the nucleus becomes homogenous, the nuclear membrane thickens, and the rotation of the nucleus ceases. Thereafter the cell lyses and falls away from the glass. If macrophages are present in the culture they ingest the remnants. The whole process may take place in a few minutes, but the changes may not be completed within an hour in some cells.

Plate 5. Oil immersion photomicrograph of 2 thyroid cells after one week in culture. These are derived from a primary toxic goitre, but there is no morphological difference between these and cells from other types of thyroid tissue. The number of spherical highly refractile cytoplasmic granules has increased, as in usual with age. These granules are more frequent in degenerating cultures, for example when the medium has not been changed for a week, and in infected cultures. They become vacuolated before lysis occurs.

Plate 6. Oil immersion photomicrograph of cells 10 minutes after addition of cytotoxic serum plus complement to a healthy 24-hour culture of toxic thyroid cells. The first observed change is the ease with which the mitochondria can be resolved. These bodies rapidly bunch up, become club shaped and retract into spheres which then become hollow and swell. The nuclear membrane has become denser also. The mitochondria in different cells show all stages of the characteristic changes.



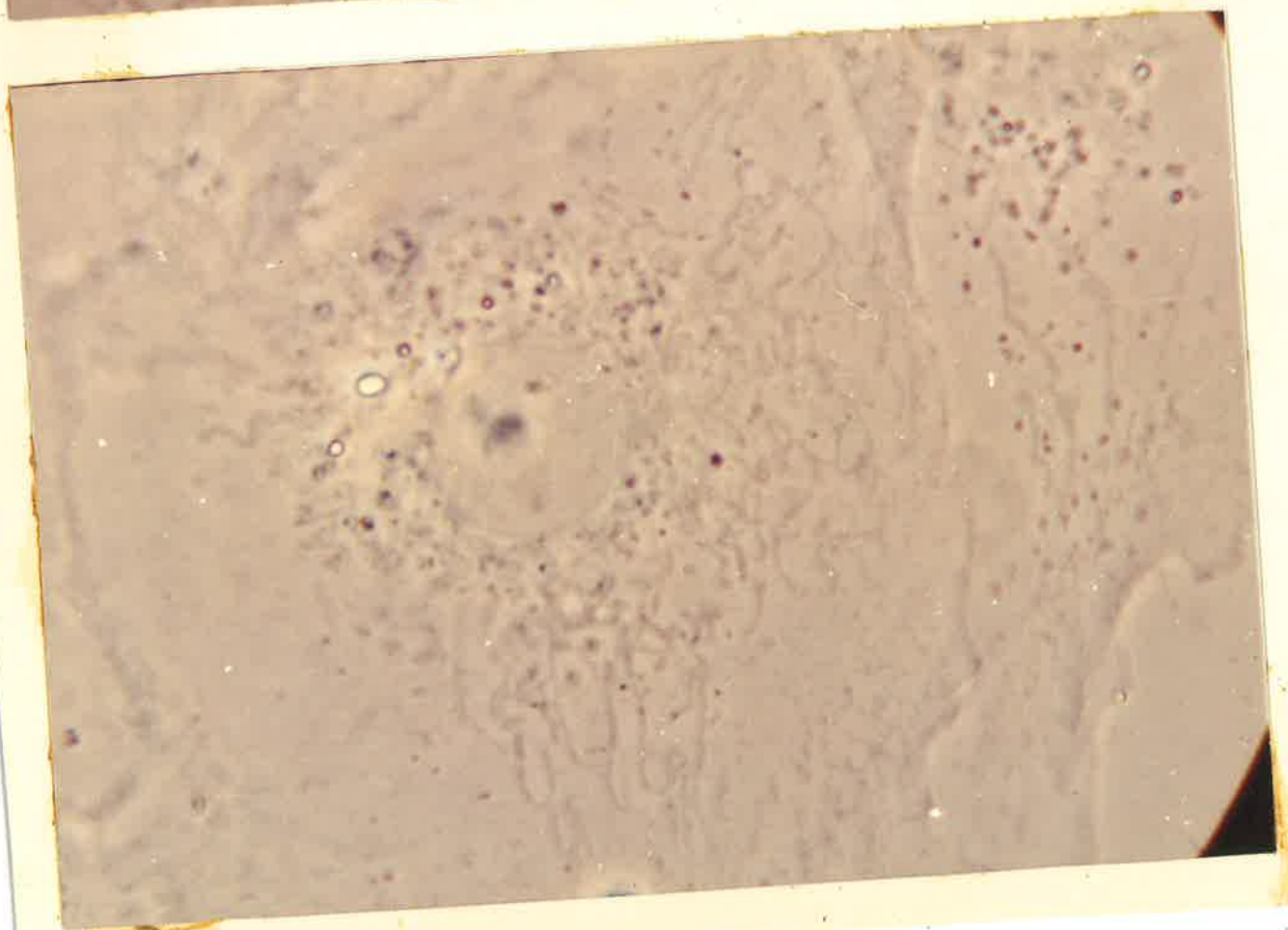
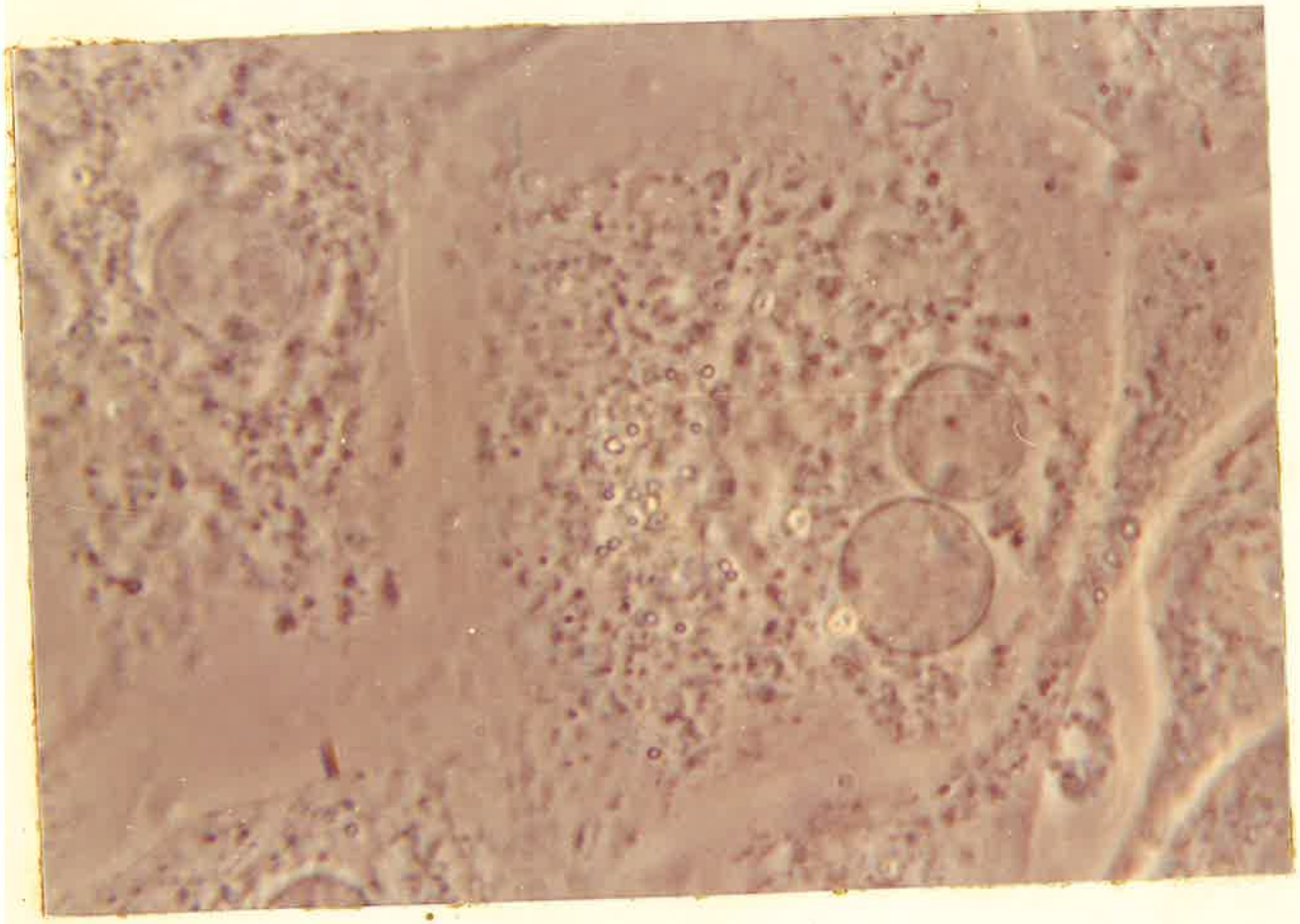


Plate 7. A slightly later stage of cytolysis than shown in the preceding plate. The central cell has two nuclei which both show the dense altered nuclear membrane and the loss of recognizable structure of the nucleus. The mitochondria have all retracted into spheres and many of them are swelling.



RESULTS

THE PART PLAYED BY COMPLEMENT

Pulvertaft et al. (1959b) in first reporting the cytotoxic action of serum from some cases of Hashimoto's disease on thyroid cells in tissue culture stated that complement did not enter into the reaction. In an experiment reported therein a strong cytotoxic effect was abolished by heating the serum to 56° C., and addition of fresh guinea pig complement did not restore activity to the heated serum.

This experiment was repeated, with some modifications, on a larger scale, and a completely opposite result was obtained. Four sera previously shown to have cytotoxic were set up as controls, and cultures were made with these sera after heating for 56° C. for 30 minutes. These heated sera were then mixed with normal unheated serum, and unheated guinea pig serum in a separate experiment.

The cultures were also set up in 50% normal serum and in 50% guinea pig serum to prove that these were not cytotoxic. 50% concentrations were used in all cultures. Where heated serum was mixed with normal or guinea pig serum, 25% of each was used.

Table 1.

Serum	Unheated	Heated	Heated + Normal	Heated + Guinea Pig
Normal	A			
Guinea Pig	A			
M	K	A	K	K
D	K	A	K	K
McD	K	A	K	K
McN	K	A	K	

To prove that complement was present in the normal and guinea pig serum a simple titration was performed on the day of the experiment using a haemolytic system. Sheep erythrocytes (Evans sheep red cells in Alsever's solution) in 6% concentration were incubated for 30 minutes at 37° C. with 10 minimal haemolytic doses (MHD) of rabbit anti-sheep erythrocyte serum. Doubling dilutions of serum were made in phosphate buffered saline in 0.1 ml. volumes, an equal volume of sensitized red cells was added, and the whole was incubated for a further 30 minutes at 37° C.

Table 2.

	1	2	4	8	16	32	64	128
Normal serum	0	0	0	0	0	1	3	4
Guinea pig serum	0	0	0	0	0	0	0	2

0 = haemolysis.
1,2,3,4 = fraction of cells not haemolysed.
(4 = no haemolysis)

It can be seen from the results shown in Table 2 that 0.1 ml. of the normal serum used contained between 16 and 32 MHD of complement.

The conclusion reached is that the cytotoxic factor is heat-stable, but requires a substance present in the unheated freshly drawn serum of the patient, or a normal individual, or a guinea pig, for its action. It is most likely that this substance is complement.

At the time this experiment was done, it was repeated by Professor Pulvertaft with identical results. In addition

complement depleted sera were prepared by Dr. I.M. Roitt by absorption of the complement on to antibody-antigen precipitates, and these were also tested by Pulvertaft, with the same results, viz. that cytotoxicity of these complement depleted sera would be restored by addition of normal sera.

The amount of complement required by cytotoxic antibody in the standard test system. A simple test was carried out, in duplicate, using 0.1 ml. of heat inactivated cytotoxic serum (C.F. titre 1/512), 0.1 ml. portions of normal human serum (containing 32 MHD of complement) or serum dilutions, and 0.6 ml. of cell inoculum. Complete lysis of toxic thyroid cells was obtained with 0.025 ml. of normal serum, but not with less, i.e., with 8 MHD.

0.1 ml. of normal human serum was therefore used routinely and was obtained for almost all of the experiments to be reported from either of two donors who had the same complement titre.

This amount of complementing serum was independently chosen by Goudie and McCallum (1962) who used chambers of the same volume and worked with 0.1 ml amounts of test serum in their studies of the thyroid cytotoxic factor.

Ross and Lepow (1960) found requirements for complementing serum in the same range in a study of cytolysis of human amnion cells in culture by rabbit anti-human amnion serum. Using a cytotoxicity index which in effect showed the maximum cytolysis which a given antibody could produce they found that 0.05 ml. of rabbit antiserum (C.F. titre 1/192) was optimally active when

0.1 ml. of normal human serum, equivalent to 5 C'H 100 units of complement, was used.

SENSITIVITY OF THYROID TISSUE TO AUTOLOGOUS ANTIBODIES

Pulvertaft and Irvine (personal communications) have both found that thyroid cells are sensitive to the patient's own thyroid antibodies. Cytotoxic factor, if present in the serum will kill autologous thyroid cells in culture, providing that the patient's cells are sensitive to homologous cytotoxic factor.

Autologous serum was used in the culture test with 11 thyroid glands. In each case the effect on the patient's own cells was the same as that using the patient's serum on homologous sensitive thyroid tissue, with the proviso that the cytotoxic effect was more marked in 3 cases with homologous completely sensitive cells than with partially sensitive autologous cells (Table 3).

The cytotoxic factor is therefore a true auto-antibody. The situation is unique. The Hargreaves L.E. cell phenomenon is thought to act only on damaged leucocytes (Robineaux, 1959) - it is difficult to envisage antibody action on a nucleus which is surrounded by cytoplasm and an intact cell wall.

THE SENSITIVITY OF VARIOUS TYPES OF THYROID GLAND TO THE CYTOTOXIC FACTOR.

Classification. A gland is regarded as yielding sensitive thyroid cells if it is affected by standard weakly cytotoxic sera. In the analysis the highly sensitive gland is recorded as ++.

If the number of cells was significantly reduced only by strongly cytotoxic sera, but there was no reduction in the number surviving in weak standard sera, the result is shown as +.

A gland yielding cells which were unaffected by strongly cytotoxic sera is shown in the analysis as -.

Results and conclusions. The sensitivity of the glands cultured is shown in Table 4, with details of the type of thyroid disease, and the results are summarized in Table 5.

It became apparent very soon that the homogeneous smoothly enlarged gland of primary thyrotoxicosis - Graves' disease - yielded cells which were most sensitive in culture to the cytotoxic factor, and cells from thyrotoxic patients were the only type which were consistently sensitive to this factor.

Nodular thyroid tissue from 3 thyrotoxic patients was also found to be sensitive to cytotoxic antibody, in two of these cases being quite as sensitive as tissue from glands of Graves' disease. In one patient who was euthyroid the uptake of radioactive iodine by a "hot" nodule was not suppressed by pretreatment with triiodothyronine (T_3) 120 microgrammes daily for 10 days. The cells from this gland were insensitive to cytotoxic factor. By contrast, in one of the cases of toxic nodular goitre in which the uptake of I^{131} was not suppressed by T_3 the cells were completely sensitive.

Cells derived from non-toxic nodular goitres or adenomata were either weakly sensitive or insensitive, as were cells obtained from "normal" thyroid tissue. These "normal" tissues were obtained from glands containing nodules in 3 cases. The cells from two of these were completely insensitive, and cells from the other partially sensitive. Unequivocally normal tissue was obtained from one patient during surgical exploration

for parathyroid tumour, and this tissue yielded cells which were sensitive to strong cytotoxic sera (+ sensitivity).

Only one gland from a patient with Hashimoto's disease was tested. The culture had several interesting features. Lymphocytes were extremely numerous. The thyroid cells which grew were smaller than those seen in most cultures and were derived from smaller clumps. Many individual cells, and groups of two or three, were present; these were mostly small bipolar cells. It is often impossible to distinguish individual thyroid cells from fibroblasts (Pulvertaft, personal communication). It is of course possible to be certain of the identity of thyroid cells when their progress from an acinar clump to a monolayer is observed. It is possible that many of the cells seen in the culture of the Hashimoto gland were fibroblasts. Strongly cytotoxic sera significantly reduced the number of surviving cells cultured from this gland. The cells which survived the action of cytotoxic factor were mainly small and spindle-shaped, presumably fibroblasts.

One papillary carcinoma tested grew indifferently in cytotoxic standard sera, and in its own serum, which contained cytotoxic factor. Growth in normal serum was little better, so that inability to become established in vitro was not attributable to cytotoxic factor.

Monkey thyroid (Macacus rhesus, obtained from the National Institute for Medical Research, Mill Hill, London N.W.7) was not obviously affected by strongly cytotoxic sera. This is surprising in view of the fact that microsomal fraction of monkey thyroid tissue fixed complement in the presence of sera

of patients with Hashimoto's disease (Roitt, Doniach, Wilson and Couchman, 1960).

Pulvertaft has found similar results in testing the sensitivity of various types of thyroid tissue (Pulvertaft, Doniach and Roitt, 1961). The cells of one foetal adenoma, and two of four thyroid carcinomata, were sensitive. He also found monkey thyroid tissue insensitive, and cells from Hashimoto's struma sensitive.

To assess the sensitivity of the thyroid cells more accurately serial dilutions of the same control serum were set up in 14 cultures (Table 6). The end-point of the titration was recorded as the highest dilution at which growth was less vigorous than in control chambers containing normal serum. Typical effects of the cytotoxic factor could always be seen at this dilution in a proportion of cells which had become attached to the glass before being lysed - loss of the shiny surface membrane seen in a healthy spherical cell before it has spread over the glass, formation of blebs in the surface membrane, and granular degeneration of the nucleus and cytoplasm. Even in healthy cultures some cells show this appearance, however, as there is always a proportion which does not survive. It is not claimed that the method gives a highly precise end-point, but confidence in it as being a reasonalbe guide either to the amount of cytotoxic factor or to the sensitivity of cells is justified by the following facts. 1. Duplicate titrations were carried out on two occasions, and the end-point was the same. 2. Duplicate chambers were set up in approximately the first 50 sera tested.

There were no discrepancies. (All positive sera reported in this paper were retested at least once.) 3. A good correlation was found between the titre in the complement fixation test with toxic thyroid antigen and the cytotoxic titre (see Table 35).

The data set out in Table 6 show that the cells used for testing for the presence of small amounts of cytotoxic factor should be sensitive to the standard serum when diluted at least to 1/120, and preferably to greater dilutions.

Table 3

Sensitivity of thyroid tissue to autologous antibodies

Case	Type of tissue	Sensitivity of cells	Own serum own cells	Own serum other cells	C.F.T.
1	Graves'	+ +	A	A	-
2	"	+ +	A	A	-
3	"	+ +	K	K	8
4	"	+ +	A	A	-
5	"	+ +	K	K	256
6	"	+ +	A	A	-
7	"	+ +	A	A	-
8	"	+	KA	K	64
9	Hashimoto	+	KA	K	64
10	Non-toxic Nod. Goitre	-	A	A	-
11	"	+	KA	K	-

C.F.T. = microsomal C.F. antibody in patient's own serum.

(- indicates negative in C.F. test.)

Table 4

	Clinical state of patient	Type of thyroid	Histology	Sensitivity
1.	Non-toxic nodular goitre.	Nodular goitre (1 cm. nodules)	Small and large follicles, containing variable amounts of colloid. No lymphadenoid process.	+
2.	Non-toxic nodular goitre.	Very large (540 g.) nodular colloid goitre.	Large and small follicles. Epithelium flat and cuboidal. One patch focal thyroiditis seen.	+
3.	Graves disease 2 years. B.M.R. originally +62%.	Uniform, fleshy 144 g.	Typical of thyrotoxicosis. Occasional patch of lymphocytic infiltration.	+ +
4.	Graves disease.	Uniform, fleshy 64 g.	Typical of thyrotoxicosis. No lymphadenoid foci.	+
5.	Graves disease.	Uniform, fleshy 28 g.	Typical of thyrotoxicosis. No lymphadenoid foci.	+ +

	Clinical state of patient	Type of thyroid	Histology	Sensitivity
6.	Non-toxic nodular goitre.	Nodular goitre, some fleshy, others colloid.	Follicles of variable size. Oedematous stroma. Epithelium mostly flat.	-
7.	Graves' disease.	Uniform, fleshy 58 g.	Typical of thyrotoxicosis. Focal thyroiditis present.	+ +
8.	Hashimoto's disease. No laboratory studies.	Two distinct parts. 1. Pale, firm and typical of Hashimoto's disease. 2. Red, soft and fleshy. Cultured together.	1. Most intense Hashimoto's disease. 2. Early Hashimoto's disease with intense plasma cell infiltration, colloidophagy, but architecture and epithelium preserved.	+
9.	Graves' disease.	Diffusely enlarged gland (32 g.). More fibrous than usual diffuse toxic gland.	Not available	+ +

	Clinical state of patient	Type of thyroid	Histology	Sensitivity
10.	Non-toxic nodular goitre. Retrosternal extension. No excessive I^{131} uptake in large retrosternal part.	Two parts: a) Large retrosternal nodule, pale and dense. b) Tissue with normal appearance.	a) Intercellular oedema, small follicles, epithelial giant cells. Tall epithelium. b) Flat epithelium.	+ / -
11.	Toxic nodular goitre. Scanning showed "hot" nodule.	Nodular goitre 2 cm. Brown nodules.	Cuboidal epithelium. No colloid resorption. Microfollicles frequent.	+
12.	Non-toxic nodular goitre.	Nodular goitre.	That of normal thyroid.	—
13.	Graves' disease.	Uniform, fleshy.	Typical of Graves' disease.	+ +
14.	Autonomous toxic adenoma. Activity not suppressed by T_3.	Haemorrhagic and necrotic nodule 1¼" D.	Tall active epithelium with microfollicles and little colloid.	+ +
15.	Graves' disease 2½ yrs. Carbimazole.	Uniform fleshy 40 g. with some fibrosis.	Cuboidal epithelium, moderate fibrosis.	+

Clinical state of patient		Type of thyroid	Histology	Sensitivity
16.	Graves' disease.	Uniform, fleshy.	Usual	+ +
17.	Graves' disease.	Uniform, fleshy, very large. 86 g.	Usual	+ +
18.	Graves' disease.	Uniform, fleshy, large gland.	More fibrosis than in pure Graves' disease, colloidophagy. Low epithelium.	+ +
19.	Graves' disease.	Uniform, fleshy, small gland 28 g.	Not seen.	+
20.	Graves' disease	Moderate sized gland, uniform, fleshy.	Usual histology of Graves' disease.	+ +
21.	Graves' disease	Uniform, fleshy, 35 g.	More thyroiditis than in pure Graves' disease. Active epithelium.	+
22.	Graves' disease.	Uniform, fleshy, 70 g.	Graves' disease, conspicuous lymphocytic infiltration and epithelial destruction. Askanazy cells.	+ +

	Clinical state of patient	Type of thyroid	Histology	Sensitivity
23.	Papillary carcinoma. Metastases in lymph nodes.	Small, dense primary growth, solid metastases.	Papillary carcinoma.	-
24.	Graves' disease.	Uniform, large.	Some thyroiditis, some epithelial granular change.	+ +
25.	Toxic nodular goitre	Multiple fleshy and colloid nodules.	Not seen.	+ +
26.	Graves' disease.	Uniform, fleshy.	Usual	+ +
27.	Monkey thyroid.	-	-	-
28.	Graves' disease.	Fairly uniform, fleshy tissue, but larger colloid acini visible.	Patches of thyroiditis, fairly hyperplastic but also granular epithelium. Small amount of colloid.	+
29.	Euthyroid nodular goitre.	a) Fleshy (2 cm. D) adenomata. b) Normal tissue from gland.	a) Adenoma, with micro- and macrofollicles.	- +

	Clinical state of patient	Type of thyroid	Histology	Sensitivity
30.	Papillary carcinoma.	a) Primary small, dense tissue. b) Metastasis in crest of ileum. Dense carcinoma tissue.	a) Papillary carcinoma. b) As above.	- -
31.	Graves' disease.	Fleshy tissue.	Usual	+ +
32.	Graves' disease	Fleshy tissue 56 g.	Usual	+ +
33.	Graves' disease.	"	Usual	+ +
34.	Graves' disease	Uniform, fleshy 32 g.	Not seen	+ +
35.	Non-toxic nodule.	Adenoma	Flattened epithelium, large acini.	-
		Normal tissue from isthmus.	Normal.	-
36.	Graves' disease. Mildly thyrotoxic intermittently for 8 years.	Evenly fibrosed goitre with a few 1 cm. colloid cysts.	Fibrosis, colloid nodules and microfollicles.	+ +

	Clinical state of patient	Type of thyroid	Histology	Sensitivity
37.	Single nodule, uptake of I^{131} not suppressed by T_3. Euthyroid. Classified non-toxic nodular goitre.	Encapsulated adenoma.	Colloid nodule with capsule with haemorrhagic and necrotic areas.	-
38.	Recurrent non-toxic nodular goitre.	Large (220 g.) typical nodular goitre.	Areas of haemorrhage, fibrosis and calcification.	+
39.	Graves' disease.	Typical 25 g.	Typical.	+ +
40.	Non-toxic nodular goitre.	Typical	Typical	-
41.	Recurrent Graves' disease.	Diffusely fibrous evenly enlarged gland.	Not seen.	+
42.	Graves' disease.	Typical.	Not seen.	+ +
43.	Recurrent non-toxic nodular goitre.		Not seen.	-
44.	Non-toxic nodular goitre.	Typical.	Not seen.	-

	Clinical state of patient	Type of thyroid	Histology	Sensitivity
45.	Graves' disease.	Typical.	Not seen.	+ +
46.	Euthyroid. Operation for parathyroid disease.	Normal.	Normal.	+
47.	Graves' disease.	Typical.	Not seen.	+ +
48.	Graves' disease.	Typical.	Not seen.	+ +
49.	Graves' disease.	Typical.	Not seen.	+ +

Table 5.

The sensitivity of various types of thyroid tissue to the cytotoxic factor.

	-	+	+ +
Graves disease		6	23
Autonomous nodule, patient hyperthyroid			1
Autonomous nodule, patient euthyroid	1		
Toxic nodular goitre		1	1
Non-toxic nodular goitre	5	4	
Non-toxic adenoma	2		
Hashimoto's disease		1	
Normal thyroid tissue	2	2	
Monkey thyroid	1		
Papillary carcinoma	2		
Metastasis of papillary carcinoma	1		

53 cultures from 49 glands are analysed. Tissue was divided into normal and nodular in 3 cases, and one carcinoma was cultured separately from its metastasis.

+ + signifies that the gland yielded cells sensitive to weakly cytotoxic sera.

+ signifies that the cells were affected only by strongly cytotoxic sera.

- indicates that an insignificant number of cells was affected by strongly cytotoxic sera.

Table 6.

Titre to which cytotoxic effects were detectable using a standard cytotoxic serum*

Experiment number	Cytotoxicity titre** (final serum dilutions)	Sensitivity†
15	6	+
16	3000	+ +
17	3000	+ +
18	3000	+ +
19	120	+
32	120	+ +
33	600	+ +
34	3000	+ +
36	3000	+ +
37	0	-
38	6	+
39	3000	+ +
40	0	-
41	6	+

** Sera were set up in dilutions of 6,24,120,600 and 3000. No higher dilutions were tested.

† + + Cells sensitive to weakly cytotoxic sera.

\+ Cells sensitive only to strongly cytotoxic sera.

\- Cells insensitive to strongly cytotoxic sera.

* The standard serum was from case 11, B.B., of the Hashimoto's disease series.

Table 7.

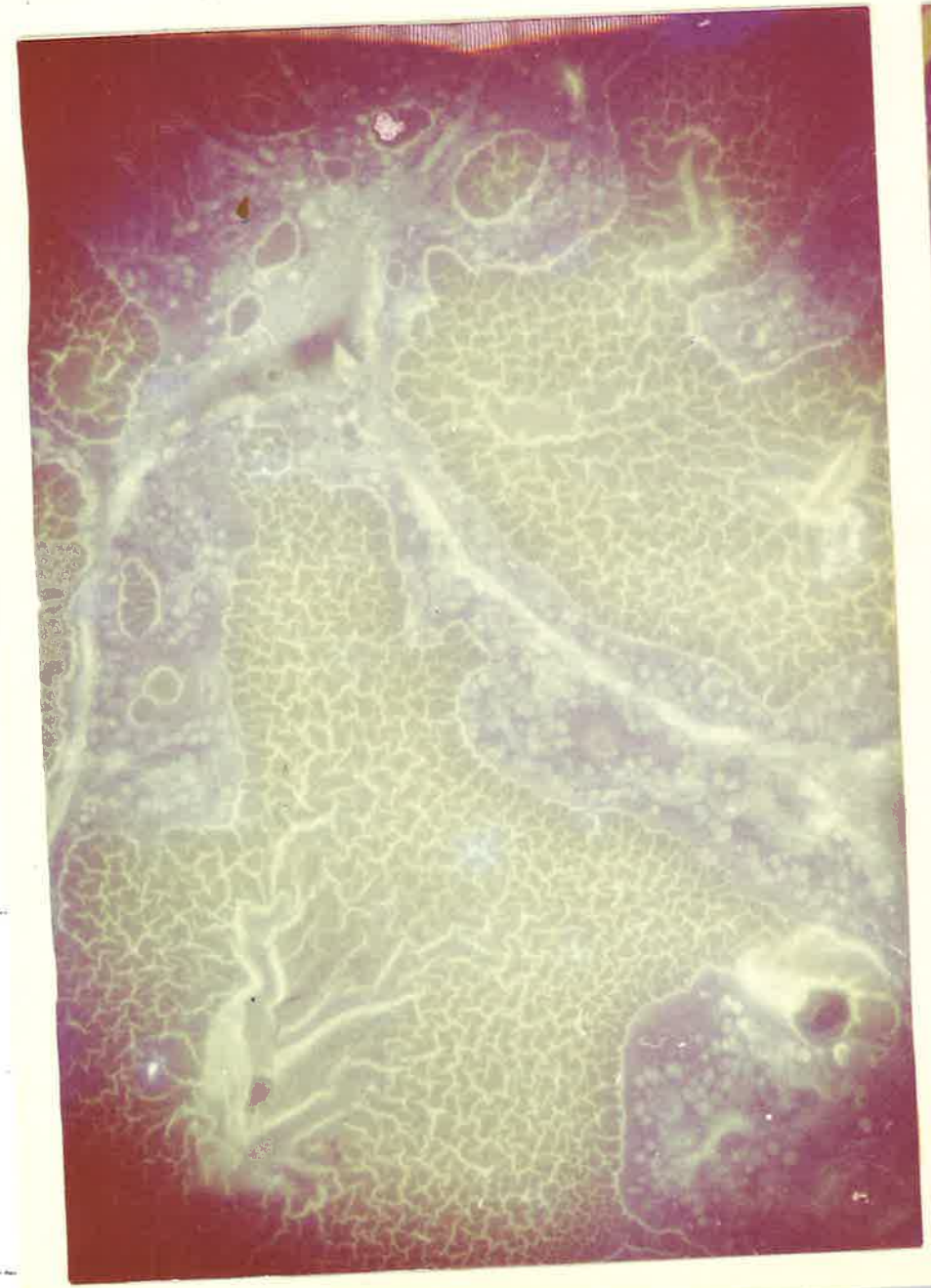
Abbreviations used in the summarised data.

K: Cytotoxic factor present.
A: Cytotoxic factor not detected.
C.F.T.: Complement fixation with toxic thyroid antigen. Figures are reciprocals of titres.
T.C.H.: Haemagglutination test with thyroglobulin - tanned erythrocytes, expressed as reciprocal of titre.
PP: Agar gel precipitin test with thyroglobulin.
Coons' colloid: Test for localization of gamma-globulin on thyroid colloid using the indirect fluorescent antibody technique.
Coons' cytoplasm: Staining of cytoplasm of thyroid epithelial cells using Coons' technique.
G: Goitre.
+P: Precipitin pattern in the above test.
+S: Homogeneous pattern in this test.
A.N.F.: Anti-nuclear factor test, detecting "staining" of nuclei by sera in the indirect fluorescent antibody technique.
N.E.: Not examined.
N: Normal thyroid function clinically and normal thyroid on palpation.
H: Hashimoto's disease.
T: Thyrotoxicosis.
F: Focal thyroiditis.
P: Propositus in genetic study.
+: Test positive.
-: Test negative.

The propositi in the genetic study were included in the thyroid disease series and are indicated by the letter "P". Four propositi not in the series are listed separately in Table 20. Conversely, the propositi to which the relatives belong are indicated by symbols denoting the case series and the number in that series, e.g., H 37 signified case number 37 in the Hashimoto's disease series.

Plate 8. Photomicrograph of brilliant fluorescence of thyroid colloid in a section prepared by the indirect fluorescent antibody technique. There is homogeneous staining of the colloid due to the localization of the antibody to the second colloid antigen. Nuclear fluorescence can also be seen.

Plate 9. The photograph shows the pattern of fluorescence seen when thyroglobulin antibody combines with the colloid. By focussing up and down it can be seen that the brilliant lines are due to the fluorescence from colloid rucked perpendicularly to the section. Nuclear fluorescence is also present.



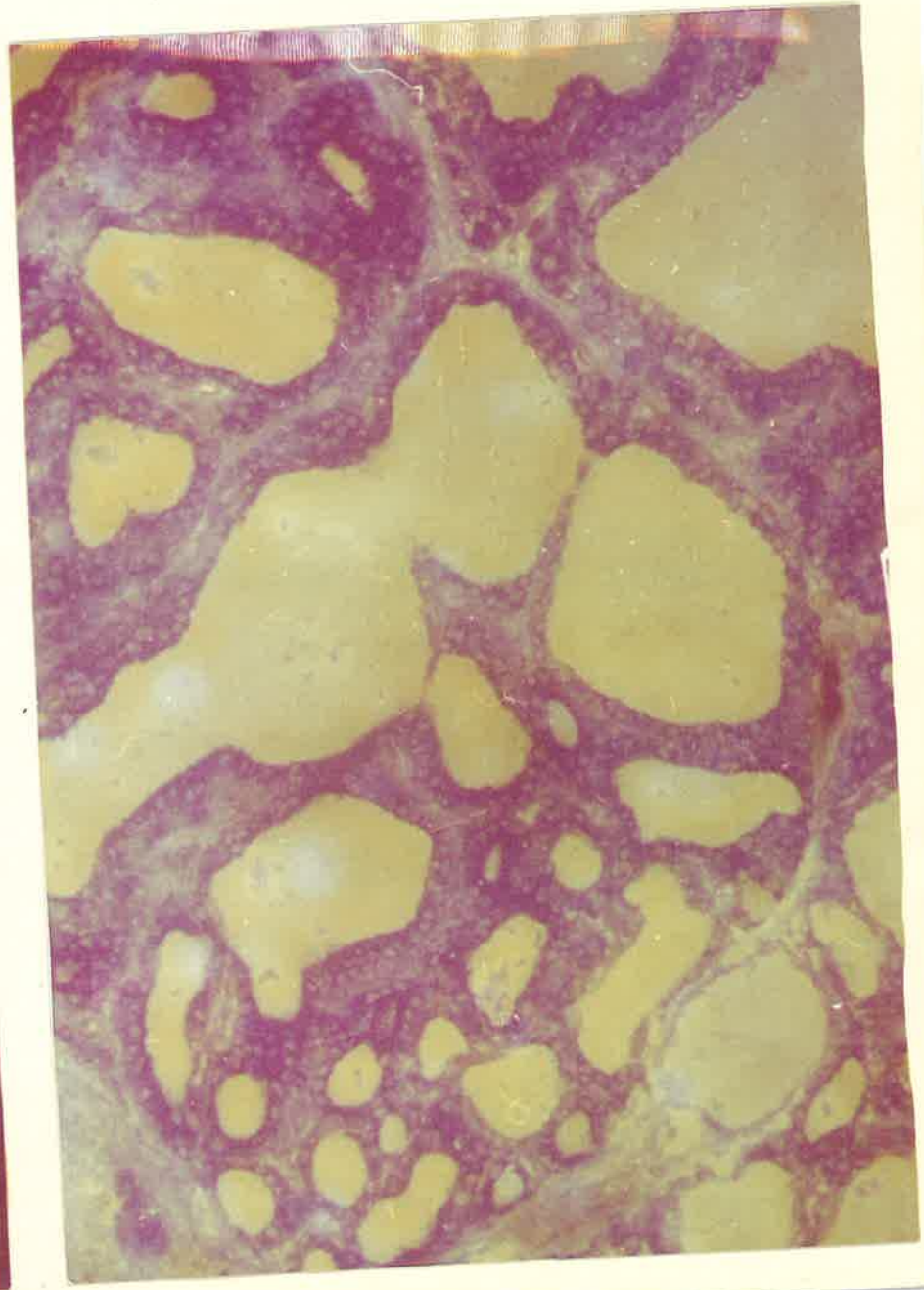
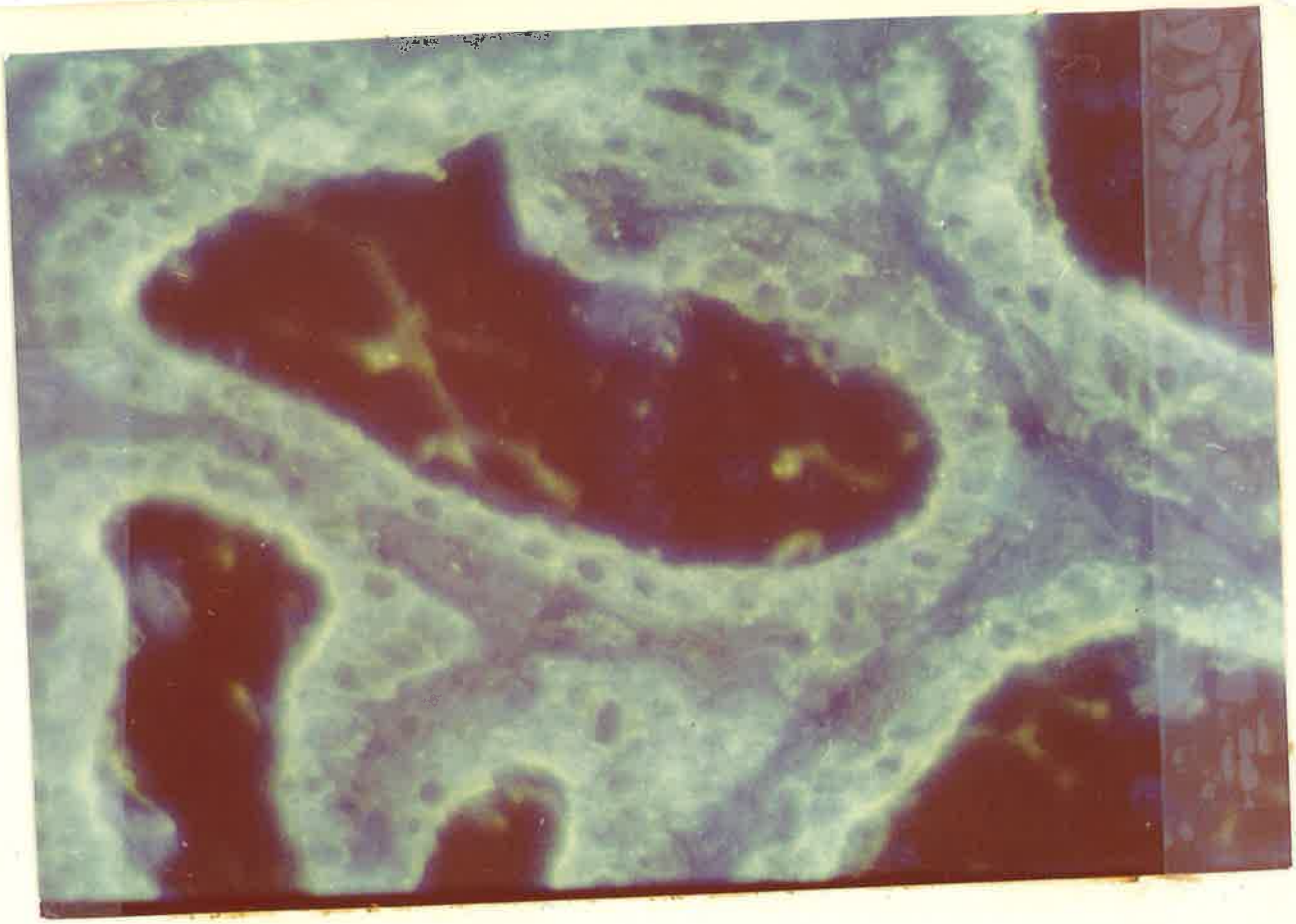


Plate 10. Cytoplasmic fluorescence. The cytoplasm of epithelial cells has stained uniformly, due to the presence of the microsomal antibody. The circles in the cytoplasm which do not fluoresce are the site of nuclei. This section was not fixed, as alcohol destroys the antigen. The colloid is washed out of unfixed sections.

Plate 11. Nuclear staining. Antinuclear factor has localized to the nuclei of thyroid cells. The serum was from a patient with Sjogren's disease who also had a goitre.



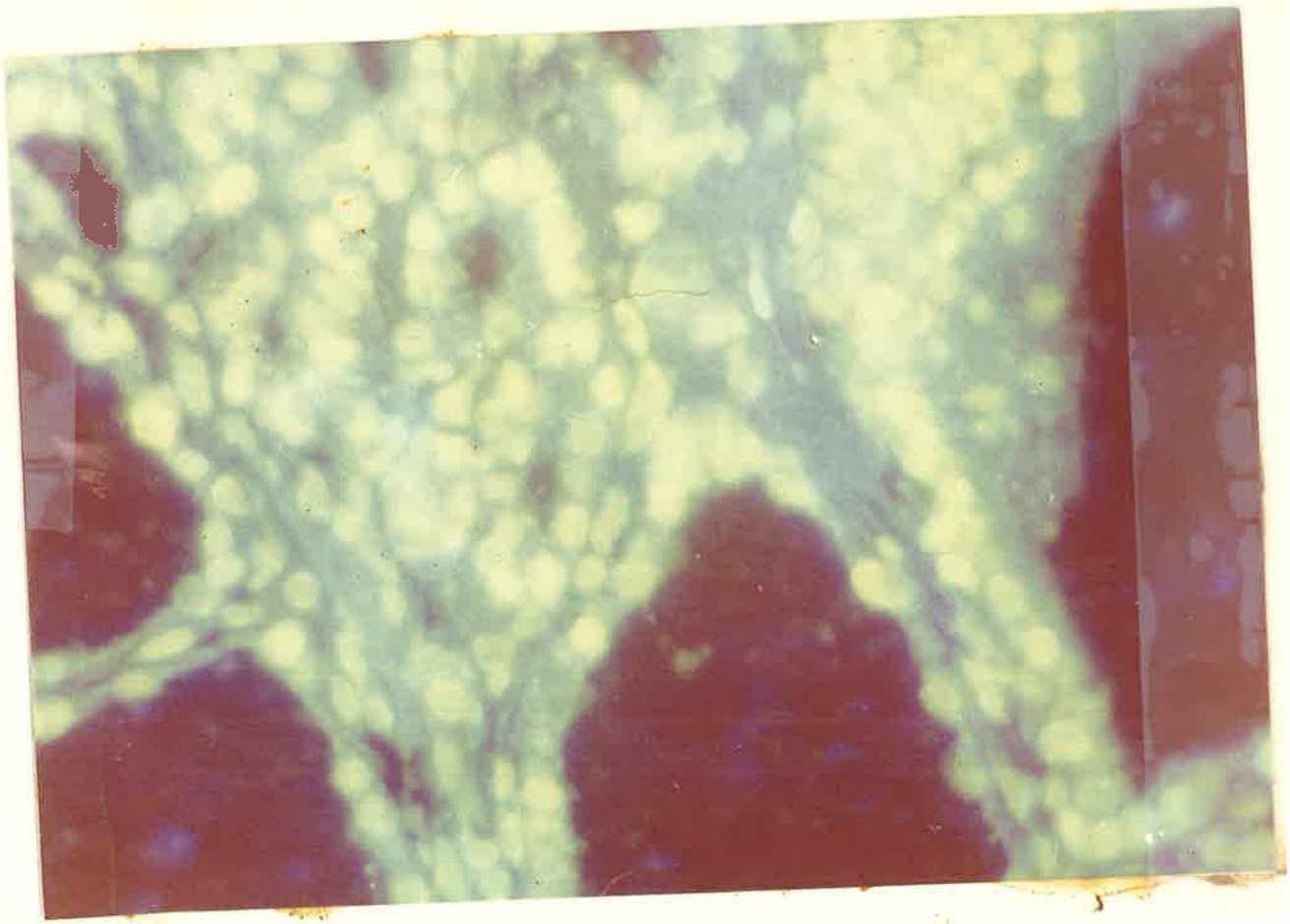


Table 8.

Hashimoto's disease

			Sex	Age	Culture	C.F.T.	PP	T.C.H.	Coons' colloid	Coons' cyto-plasm	Histo-logical proof	Response to thyroid
	1.	M.B.	F	50	K	-	-	25000		+	+	
P	2.	E.E.	F	53	K	512	+	2500000	+P	+		+
P	3.	J.L.	M	63	K	128	+	25000	+P	+		+
	4.	B.M.	F	55	K	128	-	-	+S	+		+
	5.	R.W.	M	24	K	4	-	-	+S		+	
P	6.	E.S.	F	44	K	-	-	-	+S	+	+	-
P	7.	P.B.	F	19	K	128	-	-	+S	+	+	
	8.	C.D.	F	55	K	512	-	-				
P	9.	C.H.	M	51	K	512	+	250000	+P		+	
	10.	V.H.	F	45	K	512	+	2500			+	
P	11.	B.B.	M	41	K	512	+	2500000	+P	+		+
	12.	M.H.	F	55	K	64	-	-	+S		+	
P	13.	L.L.	F	39	K	16	+	25000		+		+
P	14.	V.C.	F	50	K	64	-	-		+	+	
	15.	R.S.	F	55	K	-	+	25000		+		+
P	16.	D.G.	F	49	K	16	+	2500	+P	+		

Table 8.
(continued)

Hashimoto's disease

			Sex	Age	Culture	C.F.T.	PP	T.C.H.	Coons' colloid	Coons' cyto-plasm	Histo-logical proof	Response to thyroid
	17.	L.B.	F	53	K	512	+	2500000		+		+
P	18.	A.S.	F	64	K	256	-	-	+S	+	+	
P	19.	M.B.	F	40	K	128	+	25000		+		
P	20.	I.F.	F	62	K	256	+	250000	+P			+
P	21.	G.P.	F	56	K	512	+	2500000	+P	+	+	
P	22.	E.W.	F	55	K	-	+	250000	+P	+	+	
P	23.	G.B.	F	50	K	32	+	2500000	+P	+		
P	24.	J.L.	F	30	K	64	+	2500000	+P		+	
P	25.	F.S.	F	50	K	-	-	-	+S	+	+	
P	26.	E.S.	F	51	K	256	-	2500	+S		+	
P	27.	T.H.	F	55	K	128	-	2500		+	+	
P	28.	L.C.	F	44	K	512	-	-	+S	+	+	
P	29.	M.H.	F	39	K	512	-	-	+S		+	
P	30.	T.Q.	M	50	K	512	+	2500000	+P			+
P	31.	M.M.	F	39	K	16	-	2500		+	+	+
P	32.	I.S.	M	50	K	128	+	2500000		+	+	

Table 8
(continued)

Hashimoto's disease

			Sex	Age	Culture	C.F.T.	PP	T.C.H.	Coons' colloid	Coons' cyto-plasm	Histo-logical proof	Response to thyroid
P	33.	C.B.	F	22	K	64	-	2500	+S	+	+	
P	34.	L.C.	F	53	K	256	+	250000	+P		+	
P	35.	J.C.	F	45	K	128		250000			+	
P	36.	N.F.	F	54	K	128	+	2500000	+P			
P	37.	E.B.	F	72	A	-	+	2500000	+P	-		+
P	38.	Y.H.	F	40	K	16	+	2500000	+P			
P	39.	D.I.	F	69	K	128	-	-	+S		+	
P	40.	H.S.	M	51	K	-	+	2500000	+P	+		
P	41.	W.W.	F	49	K	-	+	2500000	+P			
P	42.	R.C.	M	67	K	-	+	2500000		+	+	
P	43.	P.C.	M	33	A	-	-	-	+S		+	
P	44.	D.T.	F	55	K	64	+	2500000				
P	45.	U.H.	F	14	K	-	-	-	+S	+	+	
P	46.	E.J.	F	64	K	16	-	2500		+		
P	47.	D.S.	F	53	K	-	-	-	+S	+	+	
P	48.	F.C.	F	53	K	-	+	2500000		+	+	

Table 8
(continued)

Hashimoto's disease

			Sex	Age	Culture	C.F.T.	PP	T.C.H.	Coons' colloid	Coons' cyto-plasm	Histo-logical proof	Response to thyroid
P	49.	B.B.	F	50	K	32	+	2500000	+P	+		
P	50.	F.W.	F	49	K	256	-	-	+S	+	+	
P	51.	S.S.	F	26	K	128	-	250000		+		
P	52.	S.F.	F	47	K	512	-	2500		+	+	
P	53.	M.S.	F	30	K	128	+	2500000	+P	+		

Table 9

Extensive focal thyroiditis

			Sex	Age	Culture	C.F.T.	PP	T.C.H.	Histological proof
P	1.	D.H.	F	29	K	128	-	-	+
	2.	D.M.	F	36	K	64	-	-	+
P	3.	S.L.	F	15	K	32	-	-	+
	4.	S.L.	F	15	K	32	-	-	+
P	5.	L.W.	F	26	K	4		250	+
	6.	A.C.	F	54	K	-	-	-	+

Table 10

Myxoedema

		Sex	Age	Culture	C.F.T.	Precipitin	T.C.H.
1.	M.K.	F		K	-	-	5
2.	L.D.	F		A	-		-
3.	S.L.	F	40	K	4		250
4.	G.D.	M	48	K	512		-
5.	M.D.	F		K	-		2500
6.	S.Y.	F	62	A	-		25
7.	A.S.	F	64	K	4	+	250000
8.	P.L.	F	28	K	-		250000
9.	S.N.	F	61	A			2500

Table 11

Thyrotoxicosis

			Sex	Age	Culture	C.F.T.	T.C.H.	
	1.	G.D.	M	22	A	-	-	Recent operation.
	2.	E.F.	F	45	K	64	250	Operation aet 13. Recurrence section 2nd op. focal thyroiditis.
	3.	H.P.	F	42	A	-	5	
	4.	H.S.	F	49	K	128	-	Carbimazole cure.
	5.	G.C.	F	50	A	-	-	Recent operation.
P	6.	F.P.	F	14	K	256	-	Carbimazole.
	7.	O.G.	F	34	K	-	2500	Operation 1 year previously.
	8.	I.O.	F	57	K	-	2500	Recent I^{131} treatment.
	9.	L.T.	F	69	A	-	-	Toxic 40 yrs. Still toxic. On neomercazole.
	10.	D.S.	F	31	A	-	-	Carbimazole.
	11.	B.	F	26	A	-	-	Operation. Post op. myxoedema.
	12.	W.H.	F	17	A	-	-	Carbimazole.
	13.	M.S.	F	34	K	128	-	Cured with carbimazole. Severe exophthalmos.
	14.	P.H.	F	41	K	256	-	Carbimazole cure. Now myxoedematous spontaneously.
	15.	E.H.	F	43	A	-	250	Post operative 3 yrs focal thyroiditis. Euthyroid

Table 11
(continued)

Thyrotoxicosis

		Sex	Age	Culture	C.F.T.	T.C.H.	
16.	M.M.	F	25	A	-	-	Operation 3 yrs previously. Euthyroid.
17.	P.F.	M	30	K	128	2500000	Carbimazole.
18.	E.H.	F	49	K	8	-	Recent operation.
19.	A.D.	F	48	K	8	2500	I^{131} treatment followed by myxoedema.
20.	A.M.	F	44	K	256	2500000	
21.	A.W.	F	63	K	-	25000	Carbimazole treatment followed by recurrence.
22.	A.M.	F	21	K	8	2500	
23.	E.M.	F	56	A	-	-	Cured by carbimazole and radiotherapy.
24.	I.W.	F	58	K	64	250000	Carbimazole cure.
25.	G.W.	M	38	K	128	25000	
26.	L.B.	F	51	K	-	-	I^{131} treated.
27.	M.L.	F	24	K	64	250	Carcimbazole cure.
28.	C.	F	47	K	32	-	
29.	E.C.	F	48	A	-	-	Operation 4 yrs ago. Focal thyroiditis. Euthyroid.

Table 11
(continued)

Thyrotoxicosis

			Sex	Age	Culture	C.F.T.	T.C.H.	
	30.	F.H.	F	26	K	64	2500	Carbimazole treatment.
	31.	E.R.	F	40	A	-	-	Hyperfunctioning adenoma.
	32.	B.J.	F	25	A	-	-	
	33.	V.M.	F	43	K	32	2500	Operation 15 yrs previously. Recurrent thyrotoxicosis. Carbimazole and I^{131} subsequently.
	34.	M.W.	F	55	K	-	25000	
	35.	S.C.	F	30	K	-	250	Operation.
	36.	E.H.	F	29	K	8	250	Operation.
	37.	I.I.	F	43	A	-	250	Post operative. Euthyroid.
	38.	M.S.	F	47	K	64	5	Now myxoedematous after I^{131} treatment.
	39.	I.P.	F	30	K	32	5	Exophthalmos.
P	40.	L.B.	F	53	K	64	-	Carbimazole, now euthyroid. Off treatment.
	41.	H.L.	F	28	K	4	250	Carbimazole, now euthyroid. Off treatment.

Table 11
(continued)

Thyrotoxicosis

		Sex	Age	Culture	C.F.T.	T.C.H.	
42.	E.J.	F	40	K	16	-	Post operative. Histology: small foci of lymphocytes.
43.	L.F.	F	76	K	64	250	
44.	P.P.	F	41	A	-	-	Recent operation.
45.	M.			K	-	-	Post I^{131} treatment.
46.	M.			A	-	-	"
47.	G.			A	-	-	"
48.	B.			A	-	-	"
49.	W.			K	-	-	"
50.	F.			K	-	-	"
51.	E.			K	-	-	"

Table 12

Non-toxic nodular goitre

		Sex	Age	Culture	C.F.T.	T.C.H.	
1.	E.D.	F	48	A	-	-	
2.	W.R.	F	40	A	-	-	
3.	A.C.	F	44	A	-	-	
4.	P.W.	F	41	A	-	-	
5.	E.F.	F	59	A	-	-	
6.	L.N.	F	71	A	-	-	
7.	N.W.	F	35	K	16	-	
8.	E.C.	F	49	A	-	-	
9.	R.S.	M	44	A	-	-	
10.	K.K.	F	54	A	-	-	Rheumatoid arthritis and Sjogren's disease.
11.	C.J.	F	48	K	-	2500	
12.	I.C.	F		A	-	-	
13.	W.H.	M	59	K	-	-	Rheumatoid arthritis
14.	B.W.	F	40	A	-	-	
15.	D.S.	F	34	A	-	25000	
16.	E.K.	F	55	A	-	-	
17.	J.E.	M	25	A	-	-	
18.	K.M.	F	36	A	-	-	
19.	A.T.	F	50	A	-	-	
20.	T.L.	F	52	A	-	-	
21.	B.L.	F	47	A	-	-	
22.	I.M.	F	53	K	128	-	

Table 12
(continued)

Non-toxic nodular goitre

		Sex	Age	Culture	C.F.T.	T.C.H.
23.	K.	F	32	A	-	-
24.	I.R.	F	42	A	-	-
25.	R.T.	M	48	A	16	-
26.	E.H.	F	58	A	-	-
27.	C.V.	F	42	A	-	
28.	N.B.	F	53	A	-	-
29.	J.F.	F	38	A	-	-
30.	D.K.	F	27	A	-	-
31.	D.N.	F	52	K	-	5

Table 13

Carcinoma of thyroid

		Sex	Age	Culture	C.F.T.	T.C.H.	Coons' colloid	A.N.F.	Coons' cyto-plasmic	Metas-tases	Type
1.	D.	F	41	K	-	-			+	+	Papillary
2.	M.J.	F	7	A	-	-			-	+	Papillary
3.	A.P.	F	74	A	-	-	-		+	+	Papillary
4.	M.H.	F	62	A		-	-	-	-		Anaplastic
5.	A.L.	F	58	A	-	-	-	+	-		
6.	A.E.	F	77	K	8	-	+S	-	+		
7.	L.H.	M		A	-	5	-	-	-		
8.	M.G.	F	40	A	-	25	-	-	+		
9.	P.	M	41	A		-	-	-	+	+	Anaplastic
10.	L.S.	F	70	A		-	+P	-	+		
11.	A.H.	F	69	A	-	-	-	-	-		

48

Table 14

Subacute thyroiditis

		Sex	Age	Culture	C.F.T.	T.C.H.	Coons' colloid	A.N.F.	Coons' cyto-plasmic
1.	P.B.	F	48	A	-	-		-	-
2.	M.C.	F	52	A	-	-		-	-
3.	M.M.	F	47	A	-	-	-	-	-
4.	M.F.	F	49	A	-	-	+S		
5.	E.K.	F	61	A	-	-			
6.	D.C.	F	47	A	-	-		-	-
7.	T.E.	F	22	A	-	-	-	-	-
8.	J.L.	F	35	K	-	25000	+S	-	+

Table 15

Thyroid antibodies in cretins and mothers of cretins

No.	Case (mother)	Culture	C.F.T.	T.C.H.	Coons' colloid	Child age (months)	Culture	C.F.T.	T.C.H.	Coons' colloid
1.	R.B.	A	-	-	-	?	A	-	-	-
2.	M.L.	K	4	-	-	?	A	-	-	-
3.	M.B.	A	-	-	+S	18	A	-	-	-
4.	H.C.	A	-	-	-	4	A	-	-	-
5.	M.D.	A	-	-	+S					
6.	W.	A	-	-	+S	3	A	-	-	-
7.	G.	A	-	-	-	7	A	-	-	-
8.	H.	A	-	-	-	8	A	-	-	-
9	D.	A	-	-	-	3	A	-	-	-
10.	S.	A	-	-	-	12	A	-	-	-
11.	S.	A	-	25	+S	?	A	-	-	-
12.	J.V.	A	-	-	-					
13.	E.S.	A	-	-	-					
14.	A.	A	A.C.*	-	-	?	A	-	-	-
15.	R.	A	16.P.F.*	-	-	?	A	-	-	-

*A.C. This serum was anticomplementary to 1/16.

*16.P.F. Partial fixation with thyroid antigen but also with liver.

Table 16

Cases of systemic lupus erythematosus tested for cytotoxic factor

	Sex	Age	Culture	C.F.T.	PP	T.C.H.	A.N.F.	L.E. cells	Thyroid status
1.	F	51	A	-		-	+		Small nodule in thyroid.
2.	F	70	A	-		-	+	+	Normal.
3.	F	40	A	32		25000	+	+	Hashi-moto's disease. (Biopsy).
4.	F	42	A	-		-	+	+	Normal.
5.	F	57	A	-		250	+	+	Normal.
6.	F	46	K	32		2500	+	+	Firm diffuse goitre.
7.	F	30	K	64	+	2500000	+	+	Hashi-moto's disease

This serum was not cytotoxic on two occasions despite the positive C.F. test. The serum fixed complement to the same titre with thyroid microsomes and with liver homogenate.

Table 17

Incidence of cytotoxic factor

Condition	No. of sera tested	Cytotoxic factor present	
Hashimoto's disease	53	51	(96%)
Focal thyroiditis	6	6	
Myxoedema	9	6	
Thyrotoxicosis	51	34	(67%)
Non-toxic nodular goitre	31	5	(16%)
Carcinoma of thyroid	11	2	
Subacute thyroiditis	8	1	
Mothers of cretins	15	1	
Cretins	12	0	
Relatives of persons having thyroid disease	118	34	(29%)
Lupus erythematosus without thyroid disease	4	0	
Lupus erythematosus with thyroid disease	3	2	
Control series	91	8	(9%)

Table 18

Incidence of thyroid antibodies in various thyroid diseases.

Condition	Number	Positive culture	Positive C.F.T.	PP	Positive T.C.H.	Coons'* colloid
Hashimoto's	53	51 (96%)	41 (77%)	28/52 (54%)	36/52 (69%)	35/36 (97%)
Focal thyroiditis	5	5	4	0/4	1/4	
Myxoedema	9	6	3/8		7	
Thyrotoxicosis	51	34 (67%)	23 (45%)		23 (45%)	
Non-toxic nodular goitre	31	5 (16%)	3 (10%)		3 (10%)	
Carcinoma of thyroid	11	2	1/8		2	2/9
Subacute thyroiditis	8	1	0		1	2/4

* Positive with either pattern.

Table 19

Genetic study

Propositi

Hashimoto's disease	45
Suspected hashimoto's disease	2
Biopsy-proven focal thyroiditis	3
Thyrotoxicosis	4
	54

Table 20

Data on propositi not included in the tables (indicated by "P")

	Sex	Age	Culture	C.F.T.	PP	T.C.H.	Coons' colloid	Coons' cyto-plasm	Diagnosis
D.W.	F	48	A	-		-	+S	-	Suspected Hashimoto
B.P.	F	30	K	16	-	25000		+	Suspected Hashimoto
L.P.	F	48	K	256		-	+S	+	Thyrotoxi-cosis
E.	F	No serological studies							Thyrotoxi-cosis

Table 21

Cytotoxic factor. Control series.

	Non-toxic	Cytotoxic
Pregnant women*	21	3
Blood donors	27	1
Various medical conditions (See "Case Material")	30	1†
Idiopathic haemolytic anaemia	1	1
Relatives of patients with S.L.E.	4	1
Normal persons	8	1
TOTAL	91	8 (9%)

* These pregnant women had healthy babies.

† Woman aged 75 with cardiovascular disease.

Table 22

Futher data on cases of the control series in which cytotoxic factor was present.

No.	Case	Sex	Age	Culture	C.F.T.	T.C.H.	Coons' colloid	Coons' cytoplasm	Condition
1.	D.R.	F	28	K	4	-	-	+	Pregnant. Thyroid just palpable.
2.	P.B.	F	31	K	-	-	-	+	Pregnant.
3.	N.M.	F	27	K	-	-	-	+	Acute haemolytic anaemia.
4.				K	-				Blood donor. No further investigations.
5.	W.	F	43	K	-	25	+S	+	Healthy mother of S.L.E. patient.
6.	B.B.	F	26	K	-	N.D.	N.D.	+	Pregnant.
7.	K.G.	F	74	K	-	N.D.	N.D.	+	Cardiovascular disease.
8.	H.S.	M	30	K	-	-	-	+	Healthy man.

There was no family history of thyroid disease in cases 1, 2, 3, 5, 6 and 8. No information was available on this point in cases 4 and 7.

Table 23

Offspring.

	Sex	Age	Culture	C.F.T.	PP	T.C.H.	Coons' colloid	A.N.F.	Coons' cyto-plasmic	Thyroid status	Propositus
1. G.P.	M	23	A	-		-	-	-	-	N	H 21
2. F.J.	M	32	A	-		-	-	-	-	N	H 46
3. M.J.	M	29	A	-		-	-	-	-	N	H 46
4. D.S.	M	11	A	-		-	-	±	-	N	H 6
5. R.S.	M	21	A	-		-	-	-	-	N	H 6
6. R.C.	M	33	A	-		250	+S	-	-	N	H 48
7. S.H.	F	26	A	-		-	-	-	-	N	L.P.*
8. N.S.	M	26	A	-		-	-	-	-	N	H 40
9. S.Q.	F	21	A	-		-	+S	-	-	N	H 30
10. E.	F		A	-		-	-	-	-	G	E*
11. V.C.	F	8	A	-		-	-	-	-	N	H 35
12. J.S.	M	31	A	-		-	-	-	-	N	H 47
13. W.S.	M	34	A	-	-	-	-	-	-	N	H 18
14. M.B.	F	25	K	-	-	25000	+P	-	+	M	H 23

*See Table 20

Table 23
(continued)

Offspring.

	Sex	Age	Culture	C.F.T.	PP	T.C.H.	Coons' colloid	A.N.F.	Coons' cyto-plasmic	Thyroid status	Propositus
15. S.B.	F	10	A	-	-	-	-			N	H 11
16. M.P.	F	25	A	-	-	-	-	-	-	N	H 21
17. J.H.	F	16	A	-		-	-	-	-	N	H 9
18. P.L.	M	18	A	-		-	-			N	H 13
19. C.P.	F	17	A	-		-	-			N	L.P.*
20. M.J.	F	28	A	-		-	-		-	N	H 2
21. K.S.	M	26	A	-		-	-			N	H 26
22. P.E.	F	31	K	16		-	+S	-	+	N	H 22
23. P.F.	M	30	K	256	-	25000		-	+	T	H 36
24. H.L.	F	28	K	4		250	-	-	+	T	T 40
25. I.H.	F	46	A	-		-	-	+	-	N	H 39
26. G.W.	M	22	A	-		-	-	-	-	N	H 50
27. N.W.	M	25	A	-		-	-	-	-	N	H 50

* See Table 20

Table 23
(continued)

Offspring.

	Sex	Age	Culture	C.F.T.	PP	T.C.H.	Coons' colloid	A.N.F.	Coons' cyto-plasmic	Thyroid status	Propositus
28. D.W.	M	28	A	-		-	-	-	-	N	H 50
29. E.W.	M	26	A	-		-	-	-	-	N	H 41
30. P.W.	F	21	A	-		-	+S	-	-	N	H 41

* See Table 20.

Table 24

Siblings.

	Sex	Age	Culture	C.F.T.	PP	T.C.H.	Coons' colloid	A.N.F.	Coons' cyto-plasmic	Thyroid status	Propositus
1. J.B.	M	60	K	64	+	2500	+P	-	+	G	H 11
2. L.F.	F	58	A	-		250		+	-	N.E.	H 11
3. E.K.	F	56	K	8		-	+S	-	+	G	H 11
4. H.K.	M	59	A	-		250	-	-	-	N	H 21
5. W.L.	F	48	A	-		-	+S	-	-	G	H 9
6. M.H.	F	50	A	-		250	+P	+	-	N.E.	H 9
7. L.H.	M	54	A	-		-	-	-	-	N	H 9
8. G.H.	M	44	A	-		-	-	-	-	N.E.	H 9
9. J.N.	F	38	A	-		-	-	-	-	N.E.	H 9
10. F.C.	M	50	A	-		-	-	-	-	G	H 28
11. W.C.	M	53	A	-		-	-	+	-	N.E.	H 28
12. G.C.	M	57	A	-		-	-	+	-	N.E.	H 28
13. C.C.	F	43	K	-		250	+S	-	+	G	H 13
14. I.M.	F	36	K	8	-	25000	+S	-	+	N	H 13

Table 24
(continued)

Siblings.

	Sex	Age	Culture	C.F.T.	PP	T.C.H.	Coons' colloid	A.N.F.	Coons' cyto-plasmic	Thyroid status	Propositus
15. S.C.	F	30	K	-		250	-		+	T	H 53
16. L.S.	F	65	A	-		-	-	-	-	N	H 20
17. N.B.	M	69	A	-		250	-	+	-	N.E.	H 20
18. A.B.	M	50	A	-		-	-	-	-	N.E.	H 20
19. A.B.	M	57	A	-		-	-	+	-	N.E.	H 20
20. S.L.	F	15	K	32		-	+S	-	+	H	F 4
21. E.S.	F	46	A	-		-	-	-	-	N	H 19
22. S.S.	F	58	A	-		-	-	+	-	N	H 26
23. J.S.	M	54	K	-		-	-	-	+	N	H 26
24. D.C.	F	55	A	-		-	+S	-	-	S	H 26
25. I.M.	F	53	K	128		-	+S	-	+	G	H 16
26. A.H.	F	50	A	-		-	-	-	-	N	H 27
27. R.R.	F	44	A	-		-	+S	-	-	G	H 34

Table 24
(continued)

Siblings.

	Sex	Age	Culture	C.F.T.	PP	T.C.H.	Coons' colloid	A.N.F.	Coons' cyto-plasmic	Thyroid status	Propositus
28. M.K.	F	50	K	8		2500		-	+	T	B.P.*
29. E.P.	M	43	A	-		-	-	-	-	G	B.P.*
30. W.S.	M	61	A	4		-	-	-	-	N	H 40
31. M.A.	F	62	A	-		-	-	-	-	N	D.W.*
32. N.C.	F	53	A	-		-	-	-	-	N	D.W.*
33. M.S.	F	47	K	64		5	-	-	+	T	H 32
34. A.I.	F	43	A	-		250	-	-	-	T	H 21
35. H.	F		K	256		2500		-	+	N	H 44
36. R.V.	F	67	A	-		25	+S	+	+	T	H 3
37. H.P.	F	61	K	-		250	+S	-	+	N	H 46
38. S.C.	M	59	K	-		-	+S	-	+	N	H 14
39. E.L.	F		A	-		-	-	-	-	N	H 7
40. L.W.	F	80	A	-		-	-	-	-	N	H 37
41. G.S.	F	45	A	-		-	-	+	-	N	H 40

* See Table 20.

Table 24
(continued)

Siblings.

	Sex	Age	Culture	C.F.T.	PP	T.C.H.	Coon's colloid	A.N.F.	Coons' cyto-plasmic	Thyroid status	Propositus
42. H.B.	M	51	K	-		-	+S	-	+	G	H 16
43. L.H.	F	74	K	-		-	-	-	+	N	H 37
44. P.H.	M	17	A	-		-	-	-	-	N	H 45
45. T.H.	M	7	A	-		-	-	-	-	N	H 45
46. P.K.	M	52	A	-		-	+S	-	-	N	H 48
47. A.L.	F	58	A	-		-	-	-	-	N	H 47
48. M.H.	F	65	A	-		-	-	-	-	N	L.P.*
49. L.B.	M	48	A	-		-	-	-	-	N.E.	H 47
50. J.B.	M	55	K	-		2500	+P	-	+	N	L.P.*
51. G.N.	M	51	A	-		-	-	-	-	N	H 36
52. D.H.	M	23	A	-		25	-	-	-	N	H 45
53. G.W.	F	50	A	-	-	-	-	-	-	N	H 47
54. M.S.	F	46	A	-		250	-	-	+	N.E.	H 35

* See Table 20.

Table 24
(continued)

Siblings.

	Sex	Age	Culture	C.F.T.	PP	T.C.H.	Coon's colloid	A.N.F.	Coons' cyto-plasmic	Thyroid status	Propositus
55. G.B.	F		A	-		-		-	-	N	H 49
56. E.L.	F	33	A	-		-	-	+	-	N	H 38
57. J.B.	M	56	A	-		-	-	-	-	N.E.	H 47
58. A.C.	F	83	A	-		-	-	-	-	N.E.	H 37
59. F.J.	F	54	K	-		-	-	-	+	N	H 36
60. H.F.	M	60	A	-	-	-	-	-	-	N	H 25
61. C.D.	M	65	A	-	-	-	-	-	-	N.E.	H 41
62. S.	F	42	K	16		-	+S	-	+	N	H 26
63. B.C.	F	23	A	-	-	-	-	-	-	N	H 43
64. T.B.	M	40	A	-		-	-	-	+	N	H 50
65. H.F.	M	45	K	-		25	+S	-	+	G	H 52
66. N.S.	M	37	A	-		-	-	-	-	N	H 51
67. J.T.	F	57	A	-		-	+S	-	-	N	L.P.*

* See Table 20.

Table 24
(continued)

Siblings.

	Sex	Age	Culture	C.F.T.	PP	T.C.H.	Coons' solloid	A.N.F.	Coons' cyto-plasmic	Thyroid status	Propositus
68. E.G.	F	63	A	-		-	-	-	-	N	H 42
69. M.M.	F	67	A	-	-	-	-	-	-	N.E.	H 41
70. E.D.	F	45	A	-	-	-	-	-	-	N.E.	H 41
71. B.D.	F	59	A	-	-	-	-	-	-	N.E.	H 41
72. E.C.	F	60	A	-	-	-	-	-	-	N.E.	H 41

* See Table 20.

Table 25

Parents

	Sex	Age	Culture	C.F.T.	PP	T.C.H.	Coon's colloid	A.N.F.	Coons' cyto-plasmic	Thyroid status	Propositus
1. F.H.	M	81	A	-		-	-	+	-	NE	H 9
2. B.B.	F	76	A	-		-	-	-	-	N	F 5
3. H.	F	53	K	-		-	+S	+	+	N	F 1
4. L.F.	F	76	K	64		250	+S	-	+	T	H 19
5. D.M.	F	60	K	16	-	2500000		-	+	G	H 24
6. A.P.	F	78	K	16		250	+S	+	+	N	H 34
7. S.P.	F	75	K	-		2500		-	+	N	B.P.*
8. M.L.	F	62	K	-		5	+S	+	+	NE	H 29
9. S.	M	86	K	16		-	-	-	+	N	H 40
10. M.P.	F	45	A	-		-	-	-	-	N	T 6
11. M.H.	F	45	K	-		-	+S	-	+	N	H 46
12. E.M.	F	58	A	-		-	-	+	-	N	H 43
13. A.D.	F	56	A	-		25	+S	-	-	N	H 7

Table 25
(continued)

Parents

	Sex	Age	Culture	C.F.T.	PP	T.C.H.	Coon's colloid	A.N.F.	Coons' cyto-plasmic	Thyroid status	Propositus
14. W.H.	M	49	K	-		-	-	-	+	N	H 46
15. V.P.	M	47	K	8		250	+S	-	+	N	T 6
16. L.J.	F	67	K	16		-	+S	-	+	N	H 51

Table 26

Incidence of all types of antibodies in relatives

	Number	Cytotoxic	C.F.T.	T.C.H.	Coons' Colloid	Cytoplasmic	Any Antibody
Offspring	30	4 (13%)	3 (10%)	4 (13%)	4S	4 (13%)	7 (23%)
Siblings	72	19 (26%)	10 (14%)	18 (25%)	16S 3P	22 (31%)	34 (47%)
Parents	16	11 (69%)	6 (37%)	7 (44%)	8S	11 (69%)	12 (75%)
TOTAL	118	34 (29%)	19 (16%)	29 (25%)	3P 28S	37 (31%)	53 (45%)

P = precipitin pattern

S = homogeneous pattern

Table 27

Incidence of cytotoxic factor. Any thyroid antibody and thyroid disease in relatives of persons having thyroid disease.

	No.	Cytotoxic factor		Any thyroid antibody		Thyroid disease	
Offspring	30	4	13%	7	23%	4	13%
Siblings	72	19	26%	34	47%	15	21%
Parents	16	11	69%	12	75%	2	12%
TOTAL	118	34	29%	53	45%	21	18%

Thyroid antibodies of one or other type were found in one or more of the relatives of 36 propositi (67%).

Thyroid disease (hyper- or hypothyroidism or goitre) was found in one or more relatives of 21 propositi (39%).

Table 28

Incidence of cytotoxic factor in 114* male and female relatives under 40 years old, and 40 years and over.

	Under 40			40 and over		
	Number	Male	Female	Number	Male	Female
Offspring	4/28 (17%)	1/15 (7%)	3/13 (23%)	0/1		0/1
Siblings	4/11 (36%)	1/5	3/6	12/58 (21%)	5/23 (22%)	7/35 (20%)
Parents				11/16 (69%)	3/4	8/12 (67%)
TOTAL	8/39 (21%)	2/20 (10%)	6/19 (32%)	23/75 (31%)	8/27 (30%)	15/48 (31%)

* Age of 4 relatives was not available in the data.

THE RELATIONSHIP OF THE THYROID CYTOTOXIC FACTOR TO OTHER THYROID ANTIBODIES

The relationship of the cytotoxic factor to other thyroid antibodies was established by performing tests for the various antibodies on a series of bloods. The details of the sera used are set out in Tables 8 through 15, and 23, 24 and 25. The data are set out in Tables 29 through 34.

Results. The data in Table 30 show that there is no close relationship between the occurrence of the second colloid antibody and the cytotoxic factor. The two antibodies are commonly present together in the same serum. (This applies to all of the thyroid antibodies.) However the cytotoxic antibody occurred in 10 sera without the other, and conversely the second colloid antibody was present in 16 sera which were not cytotoxic. A total of 165 sera were tested by both methods. The antibodies were both present in 37, and both absent in 102.

Similarly in 263 sera tested for cytotoxic factor and thyroglobulin haemagglutinating antibody, both were present in 83, thyroglobulin antibody was present alone in 13, and cytotoxic factor was present alone in 52. Both were absent in 115. High titre thyroglobulin antibody was found in two non-toxic sera (Table 31). These data again indicate that there is no absolute correlation between cytotoxic and thyroglobulin antibodies.

A closer relationship was found between cytotoxicity and the presence of microsomal C.F. antibody (Table 32). Of 90 sera positive by complement-fixation, 88 were cytotoxic. One

of the anomalous sera (Case 16 in the non-toxic nodular goitre series) giving a thyroid C.F.T. titre of 1/16 also fixed complement with a saline extract of liver to a titre of 1/16. The other anomalous serum (Case 30 in the sibling series) gave a C.F.T. titre of 1/4. Cytoplasmic antibody was absent in both of these sera. One other non-toxic serum was encountered which fixed complement to a titre of 1/32 (Case 3, systemic lupus erythematous series, Table 16). This serum is not included in Table 32. Complement-fixation also occurred with saline extract of liver to the same titre. The serum was not tested by Coons' technique. Forty-three weakly cytotoxic sera gave negative results by C.F.T. when 2 MHD of complement were used for the test. When 13 of these sera were retested with 1½ MHD of complement, 9 were positive and 4 had become anticomplementary.

The fluorescent test for cytoplasmic localization of autoantibody gave results which agreed closely with those obtained in the tissue culture test. Of 165 sera tested by both methods (Table 34), 71 were positive in both, 87 were negative by the two tests, while weak cytoplasmic staining was obtained in 6 sera in which cytotoxicity could not be demonstrated. This apparent greater sensitivity of the fluorescent antibody test is undoubtedly due to the fact that undiluted serum is used, whereas the minimal serum dilution used in the cytotoxic test is 1:8.

COMPARISON OF TITRES OBTAINED BY COMPLEMENT FIXATION, CYTOTOXIC AND CYTOPLASMIC ANTIBODY TESTS

Nineteen sera were compared by the three techniques to show that there was a correlation between the titres by each method. Methods. The complement fixation test was performed with 1½

instead of 2 MHD of complement. Serial dilutions of sera were made up in 199 medium for the tissue culture test and for the fluorescent antibody test for cytoplasmic localization. When the dilutions were used in culture, double amounts (0.2 ml.) of fresh normal human serum were used to compensate for the reduction in serum protein in the diluted test serum. Results of the fluorescent antibody tests were read and scored independently by two observers. Discrepancies were usually not greater than one dilution. When the observers differed, the lower value was taken as the end-point.

Results. The results are set out in Table 35 and Figures 2a and 2b. Considering the fact that end-points with titrations of the cytotoxic and cytoplasmic tests are not precise, the correlation coefficients of 0.848 for cytotoxic and C.F.T. titres, and 0.895 for cytotoxic and cytoplasmic titres are convincing evidence of the identity of these antibodies.

Table 29

Sera in which presence or absence of cytotoxic factor (K) and second colloid antibody (S) compared.

Series	Number	K only	S only	K+S	Neither
Hashimoto's disease	17	1	1	15	-
Subacute thyroiditis	5		1	2	2
Carcinoma of thyroid	8			1	7
Mothers of cretins	15	1	4		10
Cretins	12				12
Offspring	28	1	3	1	23
Siblings	66	5	6	11	44
Parents	14	2	1	7	4
TOTAL	165	10	16	37	102

Table 30

	Cytotoxic factor	
	Present	Absent
Second colloid antibody		
Present	37	16
Absent	10	102

Table 31

Relationship of cytotoxic factor to thyroglobulin haemagglutinating antibody.

Titre	Number	Cytotoxic Factor Present	Absent
2,500,000	21	20	1
250,000	9	9	
25,000	12	11	1
2,500	22	22	
250	21	14	7
25	5	2	3
5	6	5	1
Negative	167	52	115
TOTAL	263		

Table 32

Relationship of cytotoxic factor to microsomal complement-fixing antibody.

Titre	Number	Cytotoxic Factor Present	Absent
512	12	12	
256	9	9	
128	19	19	
64	14	14	
32	7	7	
16	16	15	1
8	9	9	
4	4	3	1
Negative	172	43	129
TOTAL	262		

Table 33

Sera on which cytotoxic (K) and cytoplasmic antibody tests were carried out in parallel.

	Number	K	Cytoplasmic antibody
Hashimoto's disease	34	34	34
Subacute thyroiditis	6	1	1
Carcinoma of thyroid	11	2	6
Offspring	26	4	7
Siblings	72	19	19
Parents	16	11	11
TOTAL	165	71	78

Table 34

Relationship of cytotoxic factor to cytoplasmic antibody.

	Cytotoxic Factor	
	Present	Absent
Cytoplasmic antibody		
Present	71	7
Absent	87	-

THE RELATIONSHIP BETWEEN MICROSOMAL ANTIGEN CONTENT OF THYROID TISSUE TO ITS SENSITIVITY TO CYTOTOXIC FACTOR

Since the concentration of the specific thyroid complement-fixing antigen is highest in toxic thyroid tissue (Roitt et al., 1960), and since cells of toxic thyroid glands are regularly sensitive to cytotoxic factor, it might be expected that the sensitivity of thyroid cells could be correlated with the antigen content of the thyroid tissue from which they were obtained. The microsomal antigen concentration of the thyroid tissue was therefore determined on 14 glands which were tested for sensitivity to cytotoxic factor in culture.

Method. The quantitative complement fixation test was based on the method of Rapport and Graf (1957). A homogenate of the whole thyroid gland was made by slicing the tissue thinly, mincing finely with scissors and disrupting in a loose fitting all glass Potter-Elvehjem homogenizer with 5 volumes of ice-cold 10% sucrose.

The fractions were tested for complement-fixing potency against a standard Hashimoto serum in the presence of three 50% haemolytic units of complement (determined by the method of Osler, Strauss and Mayer, 1952). The degree of haemolysis, measured photometrically, was plotted on a logit scale against the logarithm of the amount of antigen used for the test. The amount of antigen giving 50% haemolysis is defined as 1 unit. Controls, in which serum was omitted, were setup at all antigen dilutions in the presence of two 50% haemolytic units of complement so that any anticomplementary activity could be detected;

where this was found at antigen dilutions giving partial haemolysis in the test, the results were discarded.

The sensitivity of thyroid cells was determined according to the criteria previously stated (p.53).

Results. (Table 36, Figure 1). Two of the 3 insensitive glands tested had antigen levels below 200 units/g., and the third had a level of 500 units/g. Therefore all but one gland having an antigen content greater than 200 u./g were sensitive.

One partially sensitive gland (Culture 41) had an antigen content of 1136 units/g. whereas one very sensitive gland (culture 34) had a level of 312 units/g. The serum from this patient had a high level of microsomal complement-fixing antibody (titre 1/256). It is possible that in vivo fixation of this antibody may have neutralized the antigen to some extent, but if this were so it may be expected that sensitivity of the gland would also be low. The serum from the patient whose cells were used in Culture 41 contained C.F. antibodies at a titre of 1/64. This patient had been thyrotoxic for several years, and the gland was very fibrous. The culture may have contained some fibroblasts - a possible explanation for the low sensitivity of the gland.

Desoxyribonucleic acid content of some of the glands was determined by Dr. Roitt in order to compare better the antigen content per cell, rather than antigen content per unit weight of tissue. The relationship of cell sensitivity to complement-fixing antigen content per mg. DNA was essentially the same as the relationship of sensitivity to antigen units/g. of tissue (Figure 1).

It may be concluded that all thyroid glands containing high levels of C.F. antigen are sensitive to cytotoxic factor. However some Graves' disease glands with low antigen contents yield sensitive cells. Previous work in this laboratory (Roitt, Doniach, Hudson and Couchman, 1960) adds to this data in showing low antigen levels in non-toxic nodular goitres and normal tissues (400 units/g. or lower in 6 cases), and levels of 880, 2200 and 510 units/g. in three cases of single functioning adenoma. It has been shown that non-toxic goitres never yield highly sensitive cells, whereas "hot" nodules may. Therefore it seems certain that sensitivity is correlated with antigen content, although not absolutely.

The sensitivity of cells is probably dependent on the concentration of antigen at the surface, not on the amount within the cytoplasm.

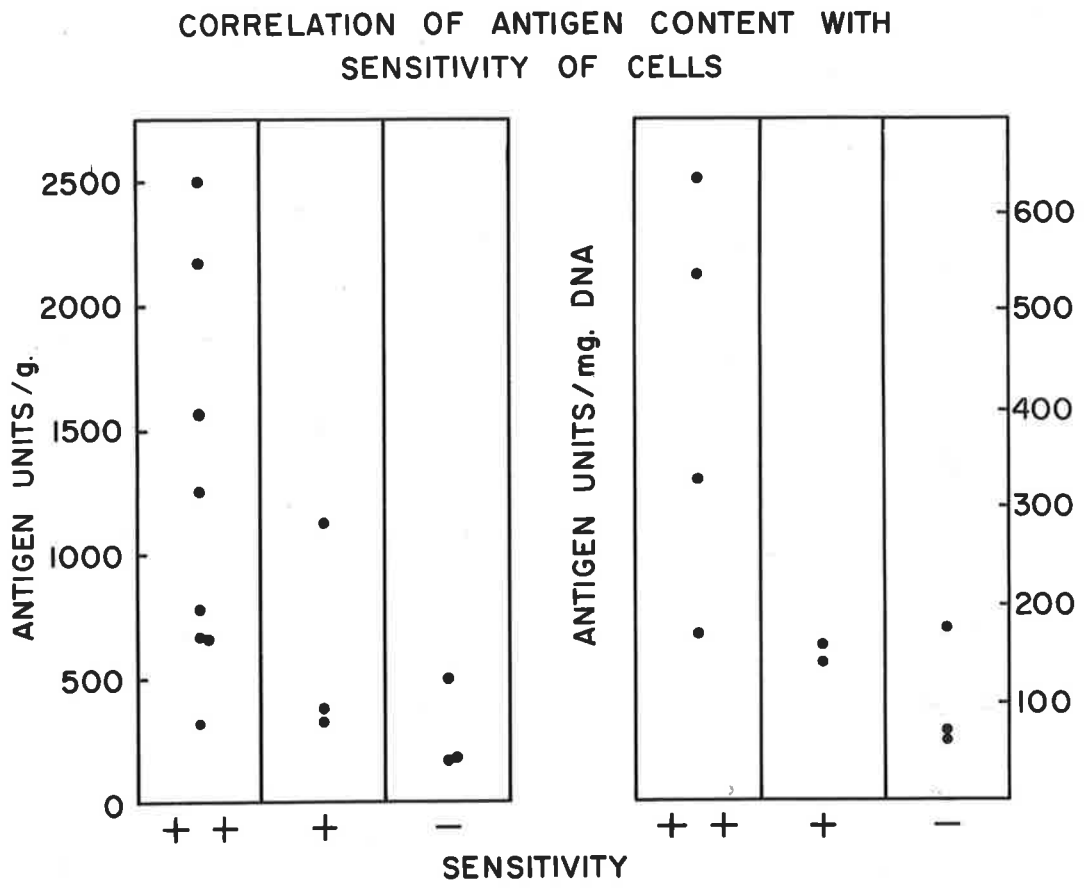


Fig. 1.: Correlation of antigen content with sensitivity of cells.

++ = sensitive to weakly cytotoxic sera.

\+ = sensitive to strongly cytotoxic sera.

\- = insensitive even to strongly cytotoxic sera.

The graphs were compiled from data given in Table 36.

Table 35

Correlation of cytotoxic, cytoplasmic and microsomal complement-fixation titres.

Serum No.	Series*	Titre† Cytotoxic	Cytoplasmic	C.F.T.
1. B.M.	H 4	3000	1000	>512
2. B.B.	H 11	3000	200	128
3. T.	-	2500	500	>512
4. L.B.	T 26	800	100	16
5. H.S.	H 40	800	100	64
6. G.D.	M 4	600	100	64
7. H.	-	120	25	32
8. R.	-	120	25	32
9. F.H.	T 30	120	8	64
10. A.W.	T 21	80	64	4*
11. J.S.	S 23	80	8	8*
12. L.	-	80	10	32
13. P.L.	M 8	80	10	128**
14. A.M.	T 22	24	8	8
15. C.J.	N 11	8	8	8*
16. M.B.	H 1	8	8	8*
17. C.C.	S 13	8	8	4*
18. M.D.	M 5	8	8	4*
19. A.D.	T 19	8	4	8

Key to symbols:*

H = Hashimoto's series.

M = Myxoedema series.

T = Thyrotoxicosis series.

N = Non-toxic nodular goitre series.

S = Sibling series.

† Figures are reciprocals of titre. C.F.T. was carried out with 1½ MHD of complement, instead of the usual 2 MHD.

* These sera failed to fix complement with toxic thyroid antigen when 2 MHD of complement were used in the test.

** This serum also fixed complement with liver extract to a titre of more than 1/512.

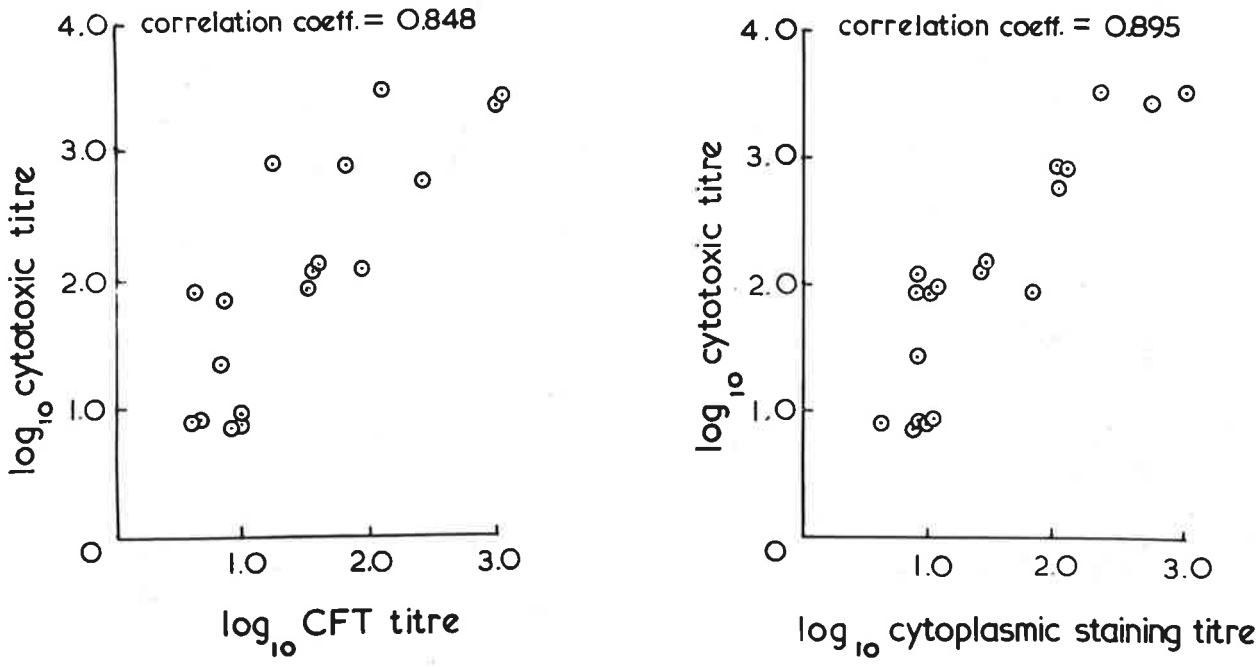


Fig. 2a.: Correlation of cytotoxicity of Hashimoto sera with complement fixing ability. The $\log_{10}$ of the reciprocal of the highest serum dilution showing cytotoxicity was plotted against $\log_{10}$ of the titre in the CFT test using 1½ M.H.D. of complement.

Fig. 2b.: Correlation of cytotoxicity of Hashimoto sera with cytoplasmic staining ability. The log10 of the titre in the cytotoxic test was plotted against $\log_{10}$ of the reciprocal of the highest dilution giving positive fluorescent staining of thyroid cytoplasm in Coon's sandwich technique.

Table 36

Relationship of microsomal antigen content to sensitivity to cytotoxic factor.

Culture	Type of gland	Sensitivity	Cytotoxic titre	Antigen units/g.	DNA mg./g.	Antigen u/mg. DNA	C.F.T. Titre of serum
25	Graves'	+ +	0	666	0	0	-
26	Graves'	+ +	0	2174	0	0	-
30	Normal	+	0	333	2.36	141	-
30	Adenoma	-	-	500	2.88	177	-
31	Graves'	+ +	0	658	3.76	175	0
32	Graves'	+ +	120	1250	0	0	-
33	Graves'	+ +	600	1562	2.60	639	0
34	Graves'	+ +	3000	312	0	0	256
36	Graves'	+ +	3000	793	2.44	325	-
37	N.T.N.G.	-	-	167	3.08	54	0
38	N.T.N.G.	+	6	395	2.44	162	0
39	Graves'	+ +	3000	2500	4.64	538	0
40	N.T.N.G.	-	-	179	2.79	64	-
41	Graves'	+	6	1136	0	0	64

- = Negative. 0 = Not done. N.T.N.G. = Non-toxic nodular goitre.

ATTEMPTS TO INCREASE THE SENSITIVITY OF INTRINSICALLY INSENSITIVE THYROID CELLS

Patients undergoing thyroidectomy for Graves' disease had invariably been treated with Lugol's solution preoperatively, and carbimazole had also been used in several instances. Experiments were therefore setup to test the sensitivity of cells from nodular goitres which were cultured in media containing high concentrations of iodine, iodide, carbimazole and thyrotropic hormone.

Method. Primary cultures were setup in the presence of potent cytotoxic sera with added complement in the usual concentrations. Increasing concentrations of the various reagents were used in cultures containing normal and cytotoxic sera. Concentrations of iodine and iodide were reached which were toxic in both normal and cytotoxic media. Below these concentrations no differences were seen. Chambers containing cytotoxic sera plus reagent were compared with chambers containing cytotoxic sera only.

Results. The following concentrations of test substances were not toxic to human thyroid cells: thyrotropin (Armour), 1.25 u. per ml.; potassium iodide, 6.25 mg. per ml.; carbimazole, 0.25 mg. per ml.; Lugol's solution to give iodine 33.3 µg. per ml.; Lugol's solution as before plus thyrotropin, 1.25 u. per ml.

In each case there was no reduction in the number of surving cells as compared with control chambers containing cytotoxic sera only.

THE EFFECTS OF ENZYMES used for cell dispersion on the characteristics of thyroid cells in culture and on their susceptibility to cytotoxic factor.

Trypsin is an endopeptidase (Rinaldini, 1958) which has a weak activity on most native proteins, but a stronger action on denatured or partially hydrolysed proteins. The enzyme is thought to remain on the cell surface and not to penetrate the cell.

Papain, obtained from the latex of Carica papaya, is a mixture of various proteolytic enzymes showing all types of exo- and endopeptidase activities on a wide variety of proteins. It digests native as well as denatured proteins. Two enzymes, named papain and chymopapain, have been crystallized from the crude extracts. They are activated by reducing agents, and inactivated by oxidising agents. Papain is effective at a lower pH range than trypsin. It has been used by Rinaldini (1958) and Hoang, Rhode and Kotschate (1959) to disperse embryonic tissues.

Thyroid tissue was not dispersed satisfactorily by papain in P.B.S., but a heavy yield was obtained with 1% papain in Parker's 199 medium.

Ficin is similar to papain in many respects (Rinaldini).

The present status of collagenase is far from clear (Rinaldini, 1958). The enzyme or enzymes are obtained from filtrates of Clostridium welchii, and there is apparently a variation in the characteristics of individual batches. Whether

it digests undenatured collagen remains to be proved. Rinaldini was unable to get satisfactory yields from various embryonic mesodermal tissues or adult cock's comb connective tissue.

However, it digests tendons, and has been found satisfactory for the preparation of thyroid cell suspensions in the present work.

Method. Papain and ficin were used as 1% solutions in 199. Collagenase was used in 0.1% strength in 199. Cultures of a gland were prepared by trypsin digestion, and separate cultures were obtained by digestion with another enzyme. The growth of trypsin-dispersed cells in media containing normal and cytotoxic sera was compared with the growth of cells obtained with another enzyme in the same sera.

Results. There were no morphological differences in free and monolayer cells obtained with ficin and collagenase. Papain was very toxic to cells when digestion was continued beyond 20 minutes. Damage to the cell membrane was visible by phase-contrast microscopy. The characteristic glistening appearance of the surface was lost. Cultures of such damaged cells failed completely.

In general the sensitivity of thyroid cells obtained by other enzymes was similar to the sensitivity of trypsin-dispersed cells. However, in one experiment cells obtained by digestion with ficin were completely insensitive to strongly cytotoxic sera, whereas the trypsin-dispersed cells were quite sensitive. A few cells regularly survived in cytotoxic sera acting on cultures obtained by the use of collagenase although the corresponding cultures of cells obtained with trypsin were completely lysed.

SEPARATION OF THE CYTOTOXIC FACTOR FROM SERUM

The cytotoxic factor has the properties of an antibody. The factor is specific for human thyroid cells, and since toxic thyroid cells are much more sensitive to the factor, it is reasonable to suggest that there is an increased concentration of an antigen in these cells. Cultures of cells from human synovial membrane, rat thyroid and monkey thyroid were unaffected. Pulvertaft found no other cells which were affected by the factor (Pulvertaft et al., 1961). It requires complement for its action. Separation of the thyroid cytotoxic factor with the gamma-globulin fraction of serum added to the evidence proving that it is an antibody.

Methods. Zone electrophoresis on "Pevikon" blocks. This was carried out by a method similar to that of Müller-Eberhard (1960). Approximately 50 g. of polyvinyl-chloride powder (Pevikon, Fosfatbolaget, Stockviksverkin, Sweden) washed three times in barbiturate buffer, pH 8.6, was made into a thick slurry and poured into a Perspex trough, 7 x 2 x ½ inches. The serum was dialyzed overnight at 4°C. against the buffer and a trace of bromophenol blue added. After equilibration of the block in the electrode chamber at 4°C. for 30 minutes, 1.5 ml. of serum was added to a depression in the block, 1 inch from the cathodal end. Electrophoresis was continued for approximately 18 hours in the cold, using a current of 7 ma until the blue-stained albumin was within 1 inch of the anodal end of the block. At the end of electrophoresis the block was cut transversely into six equal portions. The fractions were stirred with 2 ml. of Parker's 199 medium in tubes and the

supernatant recovered after centrifugation. Each fraction was dialyzed against 250 ml. of phosphate-buffered saline (PBS) at 4° C. overnight, then against 10 ml. of Parker 199 for 8 hours. The fractions were stored at -20° C until tested.

The protein fractions were identified by immunoelectrophoresis, with Scheidegger's (1955) micromethod. Four drops of each fraction in serial fivefold dilutions were set up for tissue culture; 0.2 ml. of fresh normal human serum was added to each dilution. A duplicate undiluted tube from each fraction was inactivated at 56° C. for 30 minutes before culture to ensure that cytotoxicity was complement-dependent.

Sodium sulphate precipitation of gamma-globulins from cytotoxic sera. Two vol. of 27 per cent sodium sulphate in phosphate buffer, pH 7.8, was added to 1 vol. of a cytotoxic serum, over a period of 10 minutes, with continuous stirring. After centrifugation at 10,000 r.p.m. for 10 minutes at room temperature, the supernatant was decanted and saved. The precipitate was washed in 18 per cent sodium sulphate and after centrifugation was dissolved in a volume of distilled water equal to the volume of the supernatant. The solutions were dialyzed against phosphate-buffered saline and Parker's 199 medium for use in the tissue culture test and for the detection of cytoplasmic antibodies by Coons' technique.

Chromatography of γ-globulin obtained by zone electrophoresis on Pevikon blocks. The first three fractions containing γ-globulin were used from two Pevikon blocks prepared according to the method described above. Chromatography was carried out on a diethylaminoethyl (DEAE)-cellulose column by the method of Fahey and Horbett (1959).

The Pevikon fractions were eluted in Parker's 199 medium; the final volume of solution after elution was 9.5 ml. This solution was dialyzed against an 0.01 M phosphate buffer pH 8.1, overnight.

The column was prepared with DEAE-cellulose from Serva Entwicklungslabor, Heidelberg, and washed through overnight with 0.01 M phosphate buffer. The dialyzed γ-globulin solution was added to the column and washed on with 1 ml. of buffer. A further 48 ml. of buffer was passed through the column before gradient elution was begun. The gradient was developed by running a 0.10 M phosphate buffer, pH 8.1, containing 0.35 M sodium chloride into a constant volume mixing chamber (volume 280 ml.) containing the original buffer. The eluate was collected in 4 ml. aliquots. The absorbance at 280 mμ. in a 0.5 cm. cell was measured in a Hilger spectrophotometer. The solutions were pooled into 4 fractions containing 32, 48, 40, and 130 ml., respectively. The fractions were concentrated approximately 20-fold overnight by applying a vacuum outside the solutions contained in dialysis tubing. The four concentrated fractions were dialyzed against phosphate-buffered saline and then Parker's 199 medium. All operations were carried out at 4° C. The fractions and a heat-inactivated control of each were tested for cytotoxicity in the tissue culture test.

Sucrose gradient ultracentrifugation. Five-tenths ml. of a 1:5 dilution of serum was layered above 4.5 ml. of a 12 to 36 per cent sucrose density gradient and the tubes spun at 35,000 r.p.m. in the SW 39 head of the Spinco Model L ultracentrifuge for 15 hours. After centrifugation the bottom of the tube was pierced, and separate 19S and 7S fractions were collected. The

proteins were identified by immunoelectrophoresis and tested for cytoplasmic antibody by Coons' technique. Dialyzed fractions were tested for cytotoxicity in one experiment.

Results. "Pevikon" zone electrophoresis of cytotoxic serum. Five fractionations were carried out, by which it was proved conclusively that the cytotoxic factor is contained in gamma-globulin and slow beta-globulin fractions. Portions of each fraction were used for testing by complement fixation with toxic thyroid extract in fractionations D and E, and for cytoplasmic antibody in fractionations B, D and E. In the experiments in which this was done, the complement-fixing antibody was confined to the slower moving gamma-globulin whereas cytotoxic and cytoplasmic antibody was found also in faster fractions as well.

Serum H 11 was used in these 5 experiments.

Table 37

FRACTIONATION A

Fraction	Immunoelectrophoretic analysis	Culture
1	Nil	A
2	Gamma-globulin	K
3	Gamma	K
4	Beta-gamma	K
5	Beta-globulin	K
6	Albumin alpha- and beta-globulin	K
7	Albumin alpha-globulin	A

K = complete or almost complete destruction.
KA = cytotoxic effects visible, but many cells surviving.
A = satisfactory survival of cells with spread.

The result of fractionation A indicates that the factor moves with the beta- and gamma-globulins on electrophoresis.

Table 38

FRACTIONATION B

Fraction	Immuno-electrophoretic analysis	Culture	Cyto-plasmic antibody	Culture after redialysis
1	Nil	K	+	KA
2	Gamma-globulin	K	+	KA
3	Gamma-globulin	A	+	KA
4	Gamma-globulin	K	+	KA
5	Beta-globulin	K	o	KA
6	Beta-globulin	K	-	A
7	Albumin, alpha, beta	A	-	A
8	Albumin, alpha, globulin	A	-	A
9	Nil	KA	-	A

o = not tested.

The result of fractionation B has three discrepancies. In fraction #1 no gamma-globulin was detectable by immunoelectrophoresis, but cytoplasmic antibody was present. Fraction #3 was not cytotoxic, despite the presence of cytoplasmic antibody. Cytotoxicity, apparently non-specific, was removed from fraction #9 by repeating the dialysis. At the same time the potency of the cytotoxic fractions was greatly reduced.

Table 39

FRACTIONATION C

Fraction	Immuno-electrophoretic analysis	Culture dilutions				After redialysis				Heated control
		1	5	50	250	1	5	25	125	
1	Gamma	KA	KA	KA	KA	KA	KA	A	A	A
2	Gamma	K	K	KA	KA	KA	KA	KA	A	A
3	Gamma, beta	KA	KA	A	A	KA	A	A	A	A
4	Beta, alpha	KA	A	A	A	A	A	A	A	A
5	Beta, alpha	A	A	A	A	A	A	A	A	A
6	Alpha, albumin	A	A	A	A	A	A	A	A	A

The result of fractionation C shows conclusively that the cytotoxic factor is mainly in the gamma globulin fraction of serum, but that some activity is found in beta-globulins. Again there was a loss of activity with redialysis.

Table 40

FRACTIONATION D

Fraction	Immuno-electro-phoretic analysis	Culture dilutions					Heated control	Cyto-plasmic antibody	C.F.T.
		1	5	25	125	625			
1	Gamma	KA	A	A	A	A	A	+	+ 1/24
2	Gamma	K	A	A	A	A	A	+	-
3	Gamma beta	K	KA	A	KA	A	A	+	-
4	Beta alpha	A	A	A	A	A	A	weakly +	-
5	Beta alpha	A	A	A	A	A	A	-	-
6	Alpha	A	A	A	A	A	A	-	-
7	Albumin	A	A	A	A	A	A	-	-
8	Nil	A	A	A	A	A	A	-	-

In fractionation D the cytotoxic antibody was present in fractions 1, 2 and 3. Fractions 1-3 showed strong cytoplasmic fluorescence and a weak fluorescence was obtained with fraction 4. Complement fixation was obtained with fraction 1 only.

Table 41

FRACTIONATION E

Fraction	Immuno-electrophoretic analysis	Culture dilutions 1	5	25	125	625	Heated control	Cytoplasmic antibody titre†	C.F.T.
1	γ-Globulin	K	K	KA	A	A	A	10	1/32
2	γ-Globulin	K	K	KA	KA	A	A	50	1/16
3	$\gamma + \beta_2$A-Globulin	K	KA	A	A	A	A	10	-
4	$\alpha_2 + \beta$-Globulin	KA	A	A	A	A	A	1	-
5	$\alpha_1 + \alpha_2$-Globulin	A	A	A	A	A	A	-	-
6	α_1 Globulin + albumin	A	A	A	A	A	A	-	-

† Titre given as reciprocal of highest dilution of fraction giving positive staining in relation to control section treated with negative serum.

Results in fractionation D were reproduced in fractionation E.

Chromatography of gamma-globulin obtained by zone electrophoresis on "Pevikon" blocks. Fractions 1, 2 and 3, containing gamma-globulins, were pooled from 2 blocks.

Cytotoxic activity was recovered in the breakthrough peak containing 7S gamma-globulin, while no activity could be shown in the other fractions (Figure 3, Table 42).

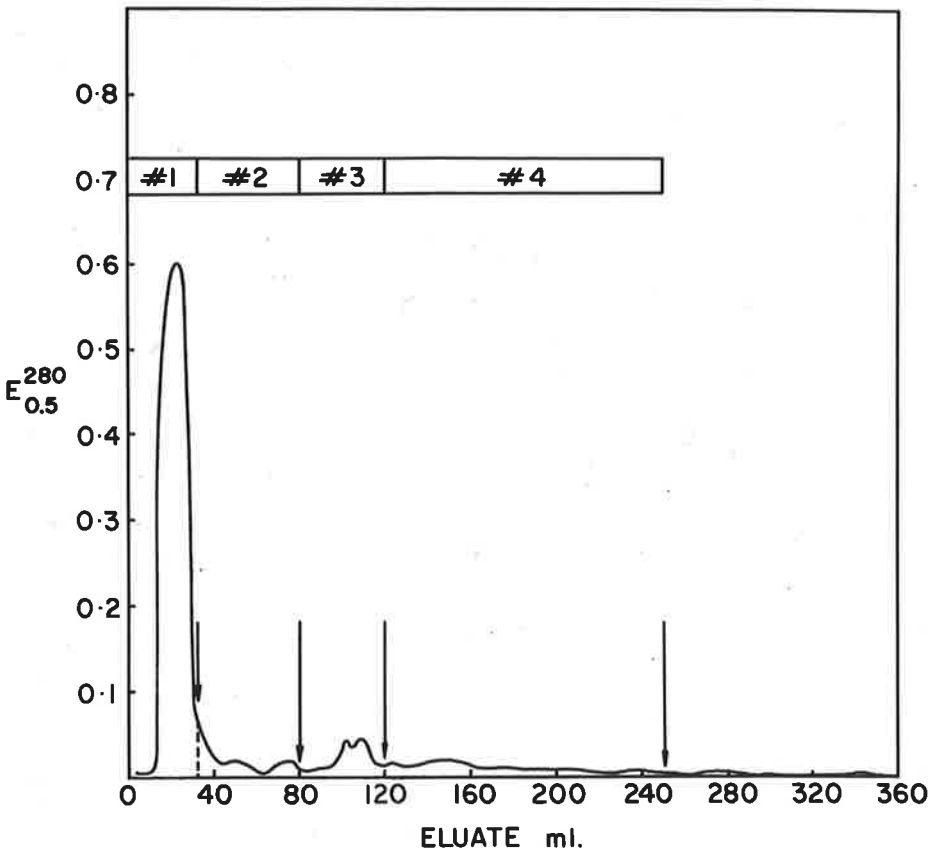


Fig. 3.: Chromatography on DEAE-cellulose columns of ɣ-globulin from cytotoxic serum obtained by zone electrophoresis on "Pevikon" blocks. Pools of eluate used in testing for cytotoxic factor in tissue culture: 1, 2, 3, 4.

Table 42

CYTOTOXICITY OF FRACTIONS OBTAINED BY DEAE COLUMN CHROMATOGRAPHY

Fraction	Culture 1	5	25	125	625	Heated control
1	K	K	KA	KA	A	A
2	A	A	A	A	A	A
3	A	A	A	A	A	A
4	A	A	A	A	A	A

Separation of gamma-globulin by sodium sulphate precipitation from cytotoxic sera. The gamma-globulin of 3 strongly cytotoxic sera was prepared by precipitation in 27% sodium sulphate. The supernatant was saved, and both fractions were dialyzed for cytotoxicity tests.

In two cases both the gamma-globulin and the supernatant fractions were cytotoxic, whereas in another the cytotoxic activity was found only in the precipitated fraction.

Table 43

SODIUM SULPHATE PRECIPITATION OF GAMMA-GLOBULIN

	Gamma-globulin	Non globulin
Serum H 11		
Cytotoxic antibody	K	A
Cytoplasmic	+	-

(Table continued next page)

Table 43
(continued)

	Gamma-globulin	Non globulin
Serum H 7		
Cytotoxic antibody	K	KA
Cytoplasmic	+	weakly +
Serum H 18		
Cytotoxic antibody	K	K
Immunoelectrophoresis	Gamma-globulin	No gamma-globulin

Sucrose gradient centrifugation. Five sera were fractionated by sucrose gradient centrifugation, and in each case cytoplasmic staining properties were recovered in the 7S globulins, while no activity could be shown in the 19S fractions. In one experiment using dialyzed fractions in the tissue culture test, cytotoxicity was contained in the 7S globulins.

FLUORESCENT TESTS ON THYROID CELLS IN CULTURE

Fluorescein-conjugated Hashimoto serum was left in contact with living susceptible monolayers at 37° C. for 45 minutes. There was no observable change in the cells, and only a faint halo of fluorescence was seen when the culture was viewed by ultraviolet light. Addition of complement produced cytotoxic changes showing that conjugated serum was still active. When monolayers from toxic and non-toxic glands were washed briefly and allowed to dry, the cytoplasm was weakly stained by conjugated globulins from a Hashimoto serum, while no fluorescence was observed with a conjugated normal serum. When conjugated antihuman-γ-globulin serum was applied after untreated Hashimoto serum (sandwich technique), the dried cells showed bright fluorescence. Far weaker staining of the cell cytoplasm was seen with normal controls, although some non-specific uptake of γ-globulin occurred.

Cultures grown in normal serum were tested daily for susceptibility to cytotoxic factor, and the cytoplasmic staining of dried monolayers was compared (Table 44). Cytotoxic serum and complement were applied to living cells and incubated for 30 minutes. Characteristic cytolytic changes were seen in 24-hour old monolayers - i.e., condensation and vesiculation of mitochondria, increased density of the nuclear membrane, loss of nuclear structure with final swelling, and rupture of the cells. A few cells remained sensitive to 48-hour old monolayers, but no effects were obtained in older cultures.

Fluorescent staining was more specific with direct Hashimoto

conjugates, and fluorescence was maximal at 24 hours, decreasing thereafter. The sandwich technique gave bright staining up to 48 hours and then decreased, but some staining was also seen with normal serum in controls.

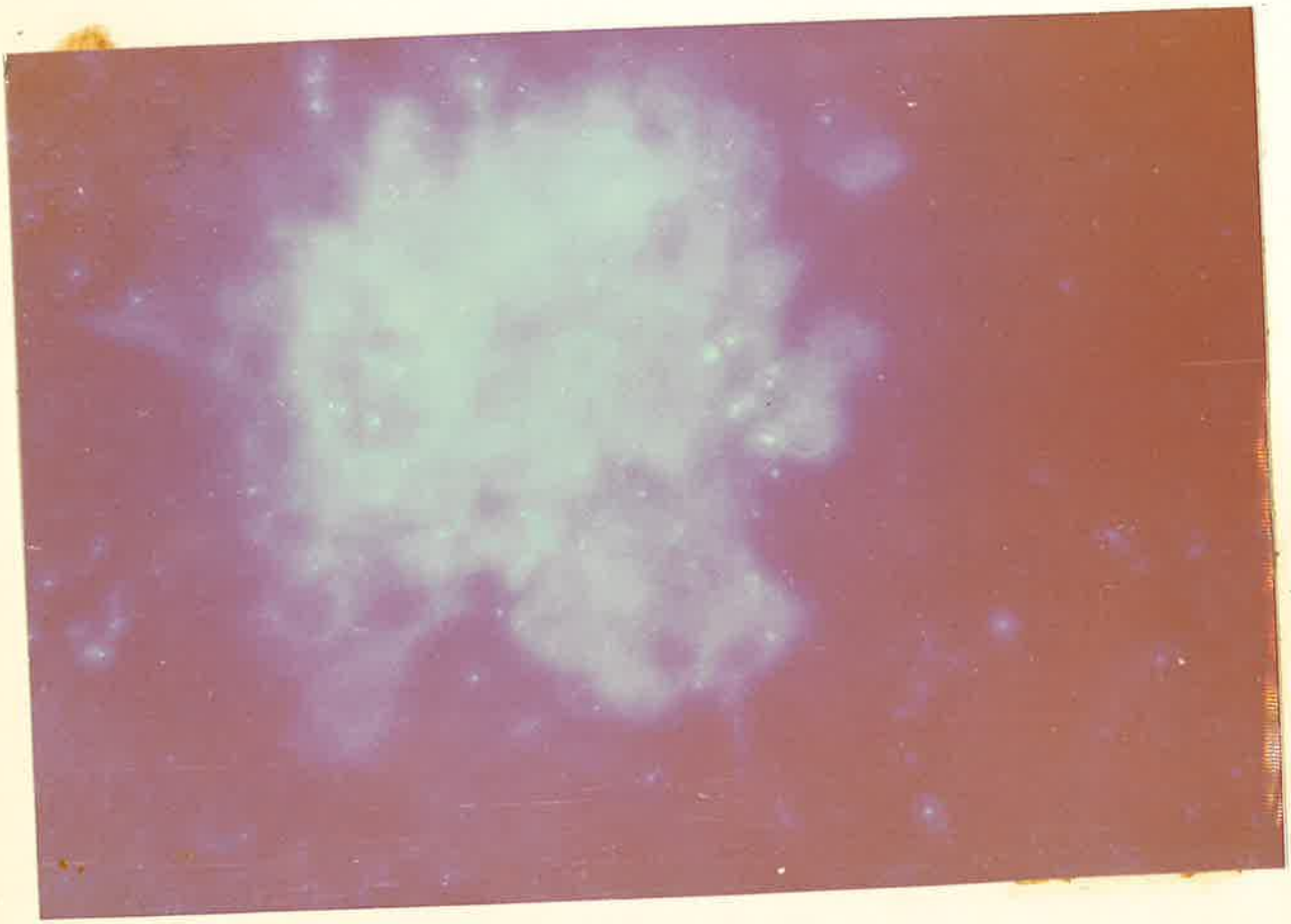
Table 44

COMPARISON OF SENSITIVITY OF THYROTOXIC CELLS IN CULTURE WITH CYTOPLASMIC STAINING OF DRIED MONOLAYERS AT 24-HOUR INTERVALS*

	Age of culture (hours)			
	24	48	72	96
Sensitivity	+	±	-	-
Direct Hashimoto conjugate	+	±	±	±
Direct normal conjugate	-	-	-	-
Sandwich technique, Hashimoto serum	+ +	+ +	+	+
Sandwich technique, normal serum	±	±	+	not done
Rabbit antiglobulin conjugate	±	±	±	±

* Strong cytoplasmic fluorescence, + +; weak cytoplasmic fluorescence, +; barely perceptible fluorescence, ±; no cytoplasmic fluorescence, - .

Plate 12. Cytoplasmic staining in dried toxic thyroid cells in monolayer culture. The cells were in culture for 24 hours. The indirect fluorescent antibody technique was used with serum H 11.



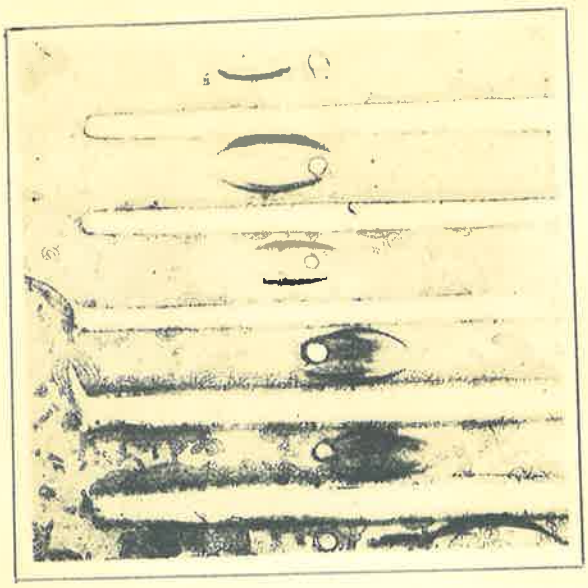


Plate 13. Photographs of immunoelectrophoretic strips of fractions obtained in fractionation E (q.v.) by zone electrophoresis of cytotoxic serum on a polyvinyl chloride block.

ABSORPTION EXPERIMENTS

a. Absorption of cytotoxic serum with whole thyroid homogenates. Method. Homogenates were prepared from toxic and non-toxic thyroid tissue by the following method: 2 cm. cubes of tissue held down by a microscopic slide were sliced as thinly as possible with a razor blade and then minced finely with scissors. The minced tissue was suspended in 10% sucrose solution in the ratio 5 ml. per gramme and homogenized in a Potter-Elvehjem blendor. Centrifugation at 650 x g. removed the coarse particulate matter.

Cytotoxic sera were mixed with homogenates of thyroid tissue and allowed to stand for 3 hours at room temperature. The suspensions were then centrifuged at 105,000 x g. for 50 minutes (Spinco, Model L, SW 39 head, 35,000 r.p.m.) to bring down all particulate elements of tissue.

The absorbed sera were dialysed in the usual way, and 0.1 ml. amounts were tested in culture for cytotoxicity, and portions were used for detection of cytoplasmic antibody by Coons' technique.

Table 45

ABSORPTION OF CYTOTOXIC SERA WITH WHOLE TOXIC THYROID HOMOGENATES

Serum +	Cytotoxic titre	Serum amount	Homogenate amount	Cytotoxic factor	Cytoplasmic antibody
1	800	0.3 ml.	2.7 ml.	A	-
2	3000	0.3 ml.	0.8 ml.	K	+
3	3000	0.3 ml.	1.4 ml.	K	+

A = Cytotoxic factor absent after absorption.
K = Cytotoxic factor present after absorption.

Results. Cytotoxic and cytoplasmic antibodies were removed from a relatively weakly cytotoxic serum by toxic whole homogenate. The amounts of homogenate used (Table 45) were not sufficient to remove these antibodies from a strongly cytotoxic serum.

The absorption experiments were repeated using sera 17 and 18 from the Hashimoto series having C.F.T. titres of 512 and 256, respectively. 0.3 ml. of each serum was absorbed with 2.7 ml. of 20% homogenate of both a toxic and non-toxic thyroid gland in the cold overnight. The toxic gland had been shown on culture to be sensitive to cytotoxic factor, and the non-toxic gland yielded insensitive cells.

The Coons' technique only was used in this experiment for testing the absorption of cytotoxic antibody, since it had become clear at this stage that cytotoxicity is never found when the cytoplasmic antibody is absent.

Table 46

ABSORPTION OF HASHIMOTO SERA WITH WHOLE HOMOGENATES OF TOXIC AND NON-TOXIC THYROID GLANDS

Serum	Absorbed with	Cytoplasmic antibody
H 17	Homogenate of non-toxic, insensitive thyroid	+
H 18		+
H 17	Homogenate of toxic sensitive thyroid	+ weak
H 18		-

Results. (Table 46.) Cytoplasmic, and therefore cytotoxic antibody, was completely removed from the weaker serum, and partially removed from the stronger by toxic thyroid, but not by non-toxic thyroid homogenate.

b. Absorption of cytotoxic sera with mitochondrial and microsomal fractions of toxic thyroid tissue.

Method. Whole homogenates were prepared in the manner previously described, and the coarse fragments of tissue and nuclei were removed by low speed centrifugation (650 x g.).

The mitochondrial fraction was sedimented by centrifugation at 7,000 g. for 17 minutes (M.S.E. refrigerated centrifuge, 8,000 r.p.m.). The mitochondrial pellet was washed in 10% sucrose and recentrifuged twice. The washings were added to the first supernatant.

The microsomal fraction was centrifuged at 105,000 x g. for 50 minutes (Spinco Model L, 40 head, 40,000 r.p.m.).

The microsomal and mitochondrial pellets were resuspended in P.B.S. using a Ten Bock homogenizer. The volumes of buffer and serum, the original amount of tissue homogenized, and the results are shown in Table 47. The fractions were mixed first with normal serum to prevent non-specific removal of cytotoxic antibody.

Results. Table 47 shows that the microsomal fraction of approximately 1.5 g. of toxic thyroid tissue in one experiment and 3 g. in another absorbed the cytotoxic factor from a weakly cytotoxic serum. In one of these experiments the mitochondrial fraction from the same tissue also removed the factor. In 4 of 5 of the

Table 47

ABSORPTION OF CYTOTOXIC SERA WITH MITOCHONDRIAL AND MICROSOMAL FRACTIONS OF SENSITIVE TOXIC THYROID GLANDS

Cytotoxic serum	C.F. titre	Amt. of test serum	Amt. of normal serum	Amt. of tissue	Suspended in	Amt. of suspension used	Cytoplasmic antibody serum absorbed with Mit	Mic
M 4	512	0.05 ml.	0.35 ml.	5 g.	8 ml.	2.6 ml.	-	-
H 28	512	0.05 ml.	0.35 ml.	5 g.	8 ml.	2.6 ml.	+	±
H 17	512	0.05 ml.	0.35 ml.	5 g.	8 ml.	2.6 ml.	+	±
M 4	512	0.2 ml.	0.6 ml.	10 g.	5 ml.	1.6 ml.	1/12	0
H 28	512	0.2 ml.	0.6 ml.	10 g.	5 ml.	1.6 ml.	1/60	1/12
-	512	0.2 ml.	0.6 ml.	10 g.	5 ml.	1.6 ml.	1/12	1/12

Mit = Mitochondrial fraction.

Mic = Microsomal fraction.

0 = Negative in test for cytoplasmic antibody.

other experiments, the microsomal fraction was more active in absorbing out the factor. The separation which is obtained by differential centrifugation is not clean (Irvine, 1962). These data however indicate that the highest concentration of the antigen with which the cytotoxic antibody reacts is in the microsomal fraction.

c. Absorption with whole trypsinized thyroid cells. Activity was absorbed from a strongly cytotoxic serum (H 11, titre 1/3000) by mixing 0.3 ml. of a 1:10 dilution of the serum with 0.2 ml. of packed trypsinized cells from a toxic thyroid gland. Absorption of the same amount of the same serum with 0.2 ml. of packed trypsinized cells from a nodular goitre did not remove the cytotoxic factor.

These experiments suggest that the antigen is also present on the cell surface.

d. Absorption of sera with thyroglobulin.

Method. Ninety mg. of lyophilized "thyroglobulin" prepared by the method of Derrien et al. (1948) was dissolved in 0.9 ml. of a standard non-toxic serum. 0.1 ml. of this (containing 10 mg. of thyroglobulin) was added to 0.1 ml. of serum to be absorbed for 2 hours at room temperature. No visible precipitate was seen. Sera were tested in this form for the presence of cytotoxic antibody, and for antibody to colloid by Coons' technique.

Results. Table 48 shows that antibodies to colloid are removed by absorption with "thyroglobulin" leaving the cytotoxic antibody. The antibody to colloid in serum 3 was certainly specific for the second colloid antigen, since no thyroglobulin antibody was detected by tanned cell haemagglutination. The antibodies

to colloid in sera 1 and 2 may have been either to thyroglobulin or to the second colloid antibody, as haemagglutinating antibodies were present.

Table 48

Serum	C.F.T.	T.C.H.	Coons' colloid	Post absorption Cytotoxic factor	Post absorption Coons' colloid
1	16	2500	+S	K	-
2	32	25	+S	K	-
3	512	-	+S	K	-

C.F.T. = reciprocal of titre of microsomal complement-fixing antibody.

T.C.H. = reciprocal of titre of thyroglobulin haemagglutinating antibody.

+S = homogeneous staining of colloid by Coons' indirect technique.

K = cytotoxic antibody present.

Lyophilised "thyroglobulin" contains both the antigen reactive in haemagglutination and the second colloid antigen. It will presumably absorb out both types of colloid antibody from serum.

CLINICAL INCIDENCE OF CYTOTOXIC FACTOR

The incidence in various conditions is presented in Table 17. Cytotoxic factor was demonstrable in all but 2 of 53 Hashimoto subjects. A high incidence was also found in patients with focal thyroiditis, myxoedema, and thyrotoxicosis. Weaker cytotoxic activity was demonstrable in a small proportion of patients with colloid goiters and thyroid carcinoma. Only 1 of 8 patients with histologically proven de Quervain's disease had cytotoxic factor. Very weak cytotoxic antibody was found in 1 of 15 mothers of athyreotic cretins and the cytoplasmic staining given by her serum was also weak.

The incidence parallels the incidence of microsomal complement fixing antibodies, but is greater because of the greater sensitivity of the tissue culture method for detecting the antibody.

Eight of 91 sera (9 per cent) tested from persons without overt thyroid disease had cytotoxic antibody. In 7 of these the antibodies were weak as judged by their inability to kill all cells in a primary culture. Stronger cytotoxic antibodies were found in a case of acute haemolytic anemia. This serum was titered on a moderately sensitive gland and was completely cytotoxic at 1/6 but did not affect the cells significantly at 1/24. Serum taken after the haemolytic process had remitted under steroid therapy was barely cytotoxic even to highly susceptible cells. Three of these positive controls were found in a group of 21 pregnant women, and they gave birth to healthy babies. The other positive controls were a 75 year old woman with cardiovascular disease, a woman with other thyroid

antibodies whose child had systemic lupus erythematosus, and two healthy subjects. Seven of the 8 cytotoxic control sera were tested for cytoplasmic staining and all gave positive results.

Thirty-four of 118 (29 per cent) close relatives of 54 persons with thyroid disease had cytotoxic antibodies. The highest incidence was found in parents (69 per cent). Twelve of the parents tested were female (cytotoxic factor in 8) and 4 were male (3 cytotoxic). The incidence in siblings and offspring was 26 and 13 per cent, respectively (Table 27).

Cytotoxic activity was present in the sera of only 2 of 7 subjects with systemic lupus erythematosus and these had associated Hashimoto's disease.

DISCUSSION

The antibody nature of the cytotoxic factor was suggested by previous work showing that complement was required for activity and that the effect was organ and species specific (Pulvertaft, Doniach, Roitt and Hudson, 1959; Pulvertaft, Doniach and Roitt, 1961). Further support for this view was obtained in the present studies by the results of serum fractionation experiments. The cytotoxic factor was found to be stable at 56°C for 30 minutes, disproving the suggestion that the antibody was heat labile (Pulvertaft et al, 1959).

The factor was precipitated together with the -globulins by salt fractionation. After zone electrophoresis, virtually all of the activity was recovered in the -globulins; the low cytotoxicity associated with the slow -globulin fraction may have been due to traces of -globulin in concentrations insufficient for detection by immunoelectrophoresis. These findings are at variance with the work of Irvine (1960) who failed to isolate the cytotoxic factor in the -globulin fraction by both paper electrophoresis and Cohn fractionation. On ultracentrifugation the factor was localized in the 7S fraction. Thus the cytotoxic factor has the properties of a normal low-molecular weight immune antibody in its ability to kill the patients' own thyroid cells in culture.

Close correlations were established between cytotoxic potency, CFT titers, and cytoplasmic staining in individual sera. The inherent difficulties of determining accurate end points in the cytotoxic and cytoplasmic staining techniques allowed for, it appears that the different tests reflect interactions of the same antigen antibody system. Activity

in the three tests is unaffected by prior absorption of the sera with thyroglobulin or second colloid antigen but is abolished by treatment with thyroid fractions known to have a high content of microsomal antigen, such as toxic thyroid gland homogenates or microsomal preparations derived from them. Irvine (1960) and Goudie and McCallum (1962) also found that cytotoxic activity was removed by extracts of toxic thyroid tissue.

The variable sensitivity of thyroid glands further accords with the view that the microsomal antigen is involved both in cytotoxic activity and in cytoplasmic staining with fluorescent antibody. Thus the most consistently sensitive cultures are derived from the glands of patients with Graves' disease, though even among thyrotoxic thyroids weakly sensitive glands were encountered. Cells from toxic nodular goiters were sensitive in three of four cases, and in two cases even to weakly cytotoxic sera. Approximately half the nontoxic nodular goiters and normal thyroid glands were unaffected by Hashimoto sera, and the remainder were only weakly sensitive.

The degree of sensitivity of a gland roughly parallels its content of microsomal CF antigen, the majority of thyrotoxic glands having a far higher content than has normal tissue (Belyavin and Trotter, 1959). The presence of an adequate level of microsomal antigen appears to be paramount in determining the sensitivity of the cells. Loss of susceptibility to the factor, which occurs in sensitive cultures after 24 to 36 hours, closely follows a marked decrease in the

staining ability of these cells in the fluorescent antibody test. This suggests that the antigen gradually disappears from the cultured cells. Leakage into the medium seems unlikely in view of the firm binding of the antigen in particulate structures within the cell (Roitt, Doniach, Wilson and Couchman, 1960). The influence of thyroxine and of thyroid-stimulating hormone on the level of CF antigen in the monkey thyroid (Roitt, unpublished results) suggests that it is related to hormonal activity; if the antigen is a hormone precursor, its continued metabolism might deplete the cell in the absence of a precursor pool in the tissue culture environment.

Denaturation of the antigen at the cell surface could also account for loss of sensitivity, and there is some evidence that the cytotoxic antibody acts on cells whose surface has been slightly modified by enzyme action. Preliminary experiments, in which minute fragments of human thyroid were cultured by the Trowell raft technique in the presence of 50 per cent Hashimoto serum with added complement, failed to show any specific tissue damage on subsequent histological examination of the fragments. Thus enzyme treatment appears to be essential for the preparation of sensitive cells, suggesting that surface digestion uncovers the required antigenic determinants. In occasional experiments, collagenase or ficin-treated cells were definitely less sensitive than were those derived from the same gland treated with trypsin.

Further evidence that the cell surface is the reacting site derives from the studies made with fluorescein-conjugated Hashimoto serum. Inability to penetrate the living cells prevented the serum from staining the cytoplasm in healthy cultures, but faint surface staining was observed. In view of the lack of penetration of antibodies into living cells, the ability of trypsinized cell suspensions to absorb out cytotoxic activity from Hashimoto serum also argues for combination occurring at the cell surface. Previous studies on immune cytolysis with other cell systems (Goldberg and Green, 1960; Hiramoto, Goldstein and Pressman, 1960; Lepow and Ross, 1960) have emphasized the importance of cell surface interactions with the antibody. Electron microscope studies using ferritin-labeled antibody to HeLa cells (Easton, Goldberg, and Green, 1961), have shown that antibody combination is restricted to the cell surface until after the addition of complement, when binding to the subcellular elements of the cytoplasmcan be observed after rupture of the cell membrane. Thus our tissue culture studies indicate that the same mechanisms obtain in autoimmune cytolysis as in heterologous immune systems.

The alteration of the cell surface apparently required for the demonstration of the cytotoxic effect makes it seem unlikely that normal thyroid cells can react with cytotoxic antibodies <u>in vivo</u>. Passive infusion into a monkey (Roitt and Doniach, 1958) of Hashimoto serum containing high levels

of cross-reacting CF antibodies indeed failed to produce thyroid injury. The fact that the cytotoxic antibodies have been demonstrated in two-thirds of thyrotoxic patients who have only a mild and nonprogressive focal thyroiditis also suggests that their presence does not imply progressive tissue destruction. However, patients with focal thyroiditis have much lower titers of cytotoxic factor than have untreated Hashimoto subjects and it may be that high-titer antibodies act synergistically with sensitized lymphoid cells. The lymphocytes may injure the cell membrane sufficiently (possibly during emperipolesis--i.e., intracellular wandering (Pulvertaft et al, 1959) to allow the cytotoxic factor to enter into the cytoplasm where it exerts its full effect. It is of interest that Robineaux (1959) was able to show by phase-contrast cinemicrography that slight surface injury of white blood corpuscles in sufficient to enable the lupus erythematosus factor to combine with live leukocytes.

The non-organ specific complement-fixing antibodies reacting with thyroid homogenates and with liver or other human and animal organs (AICF) do not produce cytotoxic effects in cultured thyroid cells, as shown by the negative results obtained with systemic lupus erythematosus and other AICF-reacting sera.

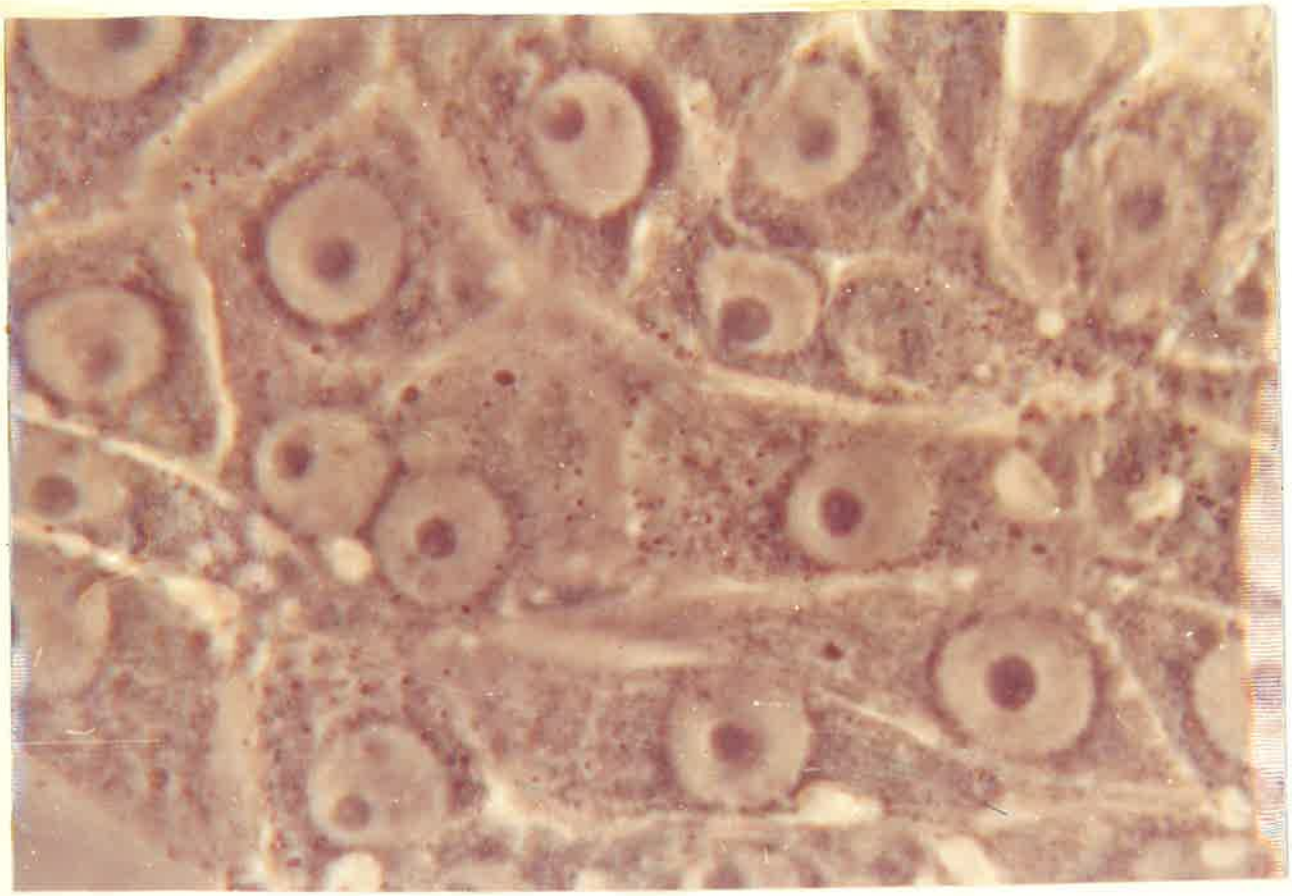
Blizzard and colleagues (1960) and Beierwaltes, Dodson and Wheeler (1959) suggested that congenital athyreotic cretinism may be due in some cases to placental transmission of cytotoxic antibodies which presumably kill the fetal thyroid during pregnancy. It was of interest to test 15

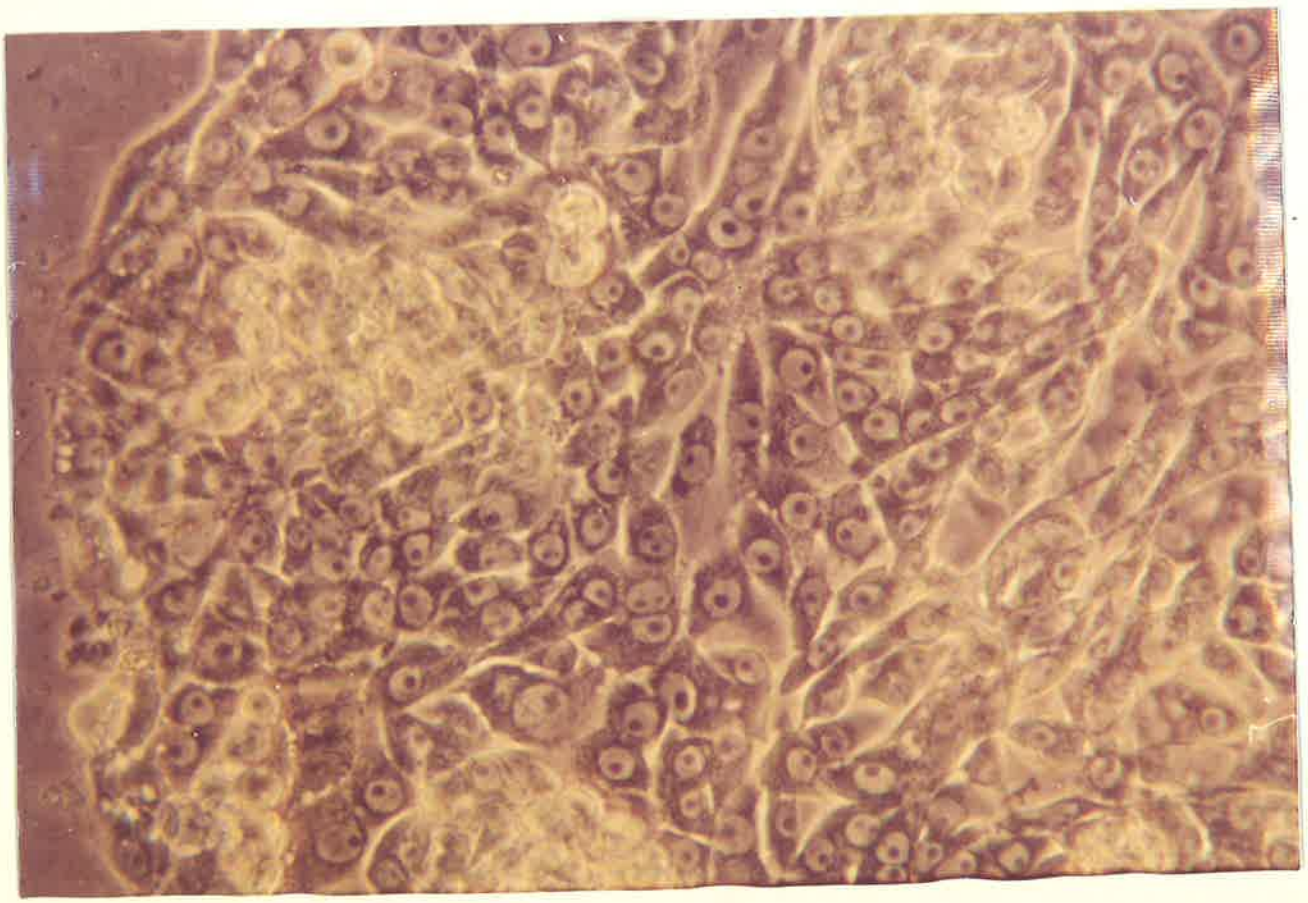
mother and cretin-baby pairs by tissue culture. Only one of the mothers' sera was weakly cytotoxic. Since similar weak cytotoxicity was demonstrated in 9 per cent of blood donors and mixed hospital patients without overt thyroid disease, the finding in this mother is not significant. Further, three mothers giving birth to healthy infants had cytotoxic antibody during pregnancy. Thus the hypothesis of placental transfer of cytotoxic antibody as a cause of cretinism receives no support from the present studies (see also Parker and Beierwaltes (1961)), although it is conceivable that sensitized lymphocytes may occasionally overcome the placental barrier and destroy the fetal thyroid.

The studies of relatives of 54 propositi with thyroid disease (mostly cases of Hashimoto's disease) confirm the data of Hall, Owen and Smart (1960), showing a strong family association of thyroid auto-antibodies. A member of such a family may have one or more thyroid antibodies, with or without detectable thyroid disease. The thyroid disease may not be the same in different members. Father, mother or both parents may have the antibodies. The figures are compatible with the expression of a dominant gene.

Plates 14 and 15. Photomicrographs of culture of metastasis of human thyroid carcinoma in the ileum.

The structure of the cells in quite different from that of normal thyroid. The mitochondria are not filamentous as in cultures of non-malignant thyroid tissue. Each nucleus has one or more very prominent nucleoli. The growth of these cells was not affected by sera which was strongly cytotoxic to non-malignant thyroid cells.





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THYROGLOBULIN ANTIBODIES IN PATIENTS WITHOUT CLINICAL DISEASE OF THE THYROID GLAND

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WORKERS from several centres have now confirmed the observations of Roitt et al. (1956) and of Witebsky et al. (1957) that autoantibodies reacting in vitro with thyroglobulin can be found in the sera of patients with thyroid disease. It is not yet established whether or not these human antibodies are autoaggressive in vivo.

The sensitive tanned-cell hæmagglutination (Witebsky et al. 1957) and complement-fixation tests using thyrotoxic antigen (Trotter et al. 1957) detect antibodies in most cases of thyroiditis and myxœdema, but also in a significant proportion of cases of thyrotoxicosis and other thyroid disorders. Although some positive results with these techniques have been reported in patients without thyroid disease (Owen and Smart 1958, Roitt and Doniach 1958, Blizzard et al. 1959, Goudie et al. 1959), it has not been suggested that such " false " or unexpected positives are frequent except in older women who may have subclinical thyroiditis of the type reported by Goudie et al. (1959).

We report here that (using the tanned-cell hæmagglutination technique) we have found thyroglobulin antibodies in the sera of 18% of 387 mixed medical hospital patients in Adelaide, in whom there was no clinical evidence of thyroid disorder. Throughout our paper we refer to these as the non-thyroid cases.

Materials and Methods

Tanned-cell Hæmagglutination Technique

Boyden's technique (Boyden 1951) using washed sheep erythrocytes was followed with the modifications introduced by Fisher (1952) and applied to the detection of antibodies specifically reacting with human thyroglobulin with which the sheep cells were coated. An antigen-inhibition control, a diluent control, an untanned-cell control, an uncoated tanned-cell control, and known positive and negative serum controls were included in each run.

Erythrocytes.—Sheep were bled into Alsever's solution and the cells used when between three and seven days old.

Thyroglobulin was prepared by the method of Derrien et al. (1948) from human glands removed at necropsy. Preliminary chessboard titrations were performed to determine the optimum concentration of antigen; this was the minimum dose giving the highest titre.

Patient's sera were stored at −20°C. Portions were removed for each test and inactivated for thirty minutes at 56°C.

Hæmagglutination Titre

'Perspex' well-plates were used for the hæmagglutinations. The reagents having been mixed, the plates were left overnight on the bench and read the following morning. Agglutination patterns were graded from the strongest to the weakest visible with the naked eye as +++, ++, +, ±, and −. ± and − were regarded as negative. Sera were put up in doubling dilutions starting at 1/10, and no serum was tested at a greater strength than this. The end-point of a titration was taken as the last tube reading +. A " positive result " was hæmagglutination at a dilution of 1/10 or higher.

Thyroid Diseases

Blood was taken in the Royal Adelaide Hospital from patients in whom a firm diagnosis of thyroid disease had been reached by the usual clinical and laboratory methods, including measurement of the basal metabolic rate, serum protein-bound iodine, and radioactive-iodine uptake. Histological confirmation was obtained only in cases where thyroidectomy was performed.

Non-thyroid Cases

Blood was taken as a routine from all patients admitted to the wards of the medical professorial unit of the Royal Adelaide Hospital. 20 sera (3 of which gave positive results) sent by colleagues from cases of diffuse lupus erythematosus, of " diffuse collagen disease ", or with abnormal serum electrophoretic patterns, in other clinical departments of the same hospital, were also included, and so there is in our material some weighting of these conditions over and above their random occurrence in the medical beds of the hospital. The whole series was obviously affected by many selecting influences, such as the special interests of consultants, and the organisation and scope of the medical services in the areas from which the hospital draws its patients, but the 387 cases were chosen before the results of the antibody tests were known.

Results

Non-thyroid Cases

The results of the tanned-cell hæmagglutination (thyroglobulin) tests in our series of 69 thyroid cases confirmed that the technique in our hands had a sensitivity comparable to that reported by other workers. Consequently, it was surprising to find that 9% of 102 blood donors and 18% of 387 hospital medical cases, chosen for their apparent freedom from thyroid disease, should give positive reactions too (table I).

We therefore reviewed all of the 68 non-thyroid cases giving positive reactions and made sure that they did not have clinical thyroid disease as ordinarily recognised. Biopsy of the thyroid glands of these patients was not justified. The diseases they had are given in table II. Positive findings were not related to recent blood-transfusion.

The 5 positive male cases in the Miscellaneous and Undiagnosed section were suffering from parkinsonism, thrombophlebitis migrans, left ventricular failure from unknown cause, pyrexia of unknown cause, and barbiturate overdosage; and the 5 females from anxiety state, fæcal impaction in a mental defective, aneurysm of the internal carotid artery, senile

TABLE I—RESULTS OF TANNED-CELL HÆMAGGLUTINATION (THYROGLOBULIN) TESTS
(The 69 thyroid cases comprised 66 females and 3 males.)

Patients	Number tested	Positives	%
Thyroid disease			
Hashimoto's thyroiditis	8	6	75
Myxœdema	20	12	60
Thyrotoxicosis	23	13	57
Euthyroid goitre	16	10	62
Carcinoma of thyroid	2	1	..
Total ..	69	42	61
Mixed medical patients without thyroid disease	387	68	18
Normal blood-donors	102	9	9

osteoporosis, and spontaneous crush fracture of the body of T8 of unknown cause.

The incidence of positives in non-thyroid cases rose with increasing age in the female (fig. 1). Divided into cases over and under 40 years old, the results were:

Over 40, males 9% of 139 cases: females 30% of 116 cases.
Under 40, males 12% of 72 cases; females 19% of 64 cases.

Antibody Titres in Thyroid and Non-thyroid Cases

One might reasonably expect to have found that the thyroid antibodies in non-thyroid cases were present in lower titre than in patients with thyroid disease. To some extent this was true (fig. 2). However, no sharp division could be made. A point about the 1 320 level would exclude three-quarters of the non-thyroid cases, but would also remove half of those patients with clinical thyroid disease.

TABLE II—NON-THYROID CASES TESTED BY THE TANNED-CELL HÆMAGGLUTINATION TECHNIQUE

Disease category	Male		Female	
	Total	Positive	Total	Positive
Syphilis	1	0	1	0
Tuberculosis, sarcoidosis	2	0	3	1
Bacterial infections	17	2	22	7*
Viral infections	21	2	14	4
Carcinoma, sarcoma	14	2*	17	7*
Reticuloses	1	0	4	0
Lymphatic leukæmia	1	0	1	0
Myelogenous leukæmia	1	0	1	0
Myelofibrosis	1	0	0	0
Para-, dys-proteinæmia	3	0	3	1*
Idiopathic acquired hæmolytic anæmia	1	0	2	1
Other anæmias	2	0	3	1
Disseminated lupus erythematosus	1	0	9	3**
Other "diffuse collagen" diseases	2	0	5	3
Rheumatoid arthritis	2	0	8	1
Asthma	6	0	2	0
Rheumatic fever	1	0	3	0
Glomerulonephritis	5	2	3	2
Nephrotic syndrome	2	0	3	0
Peptic ulcer, gastrointestinal hæmorrhage	13	0	6	1
Cirrhosis	18	3	6	2*
Acute hepatocellular disease	5	0	5	0
Diabetes	6	0	7	1
Addison's disease	0	0	1	1
Arteriosclerosis, atheroma, hypertension and sequelæ	32	6	18	5***
Miscellaneous and undiagnosed (see text)	50	5	32	5*
	208	22 (10·6%)	179	46 (25·7%)
Both sexes	Total 387 Positive 68 (17·6%)			

* See text.

All but 1 of the non-thyroid cases with titres of 1/5120 and above were women, and none was younger than 35 (average age 56). They are indicated by asterisks in table II. The conditions from which they were suffering are as follows: 1 male had carcinoma of the head of the pancreas; and the 10 females had severally shigella enteritis, carcinoma of the cervix, lymphoma with paraproteinæmia, disseminated lupus erythematosus (2 cases), active portal cirrhosis, hypertension in pregnancy, malignant hypertension, myocardial infarction, and senile osteoporosis. It may be that they had occult thyroid disease as well. On the other hand this incidence of high titres in women may mean only that they tend more than men to disease patterns involving serological disturbances, as in diffuse lupus erythematosus which is predominantly a disease of females and is associated with the appearance of irregular, exaggerated, or perhaps even irrelevant serum antibodies.

The blood donors comprised an equal number of males and females. The positive reactors (4 of whom were giving their first donation) were 2 males and 7 females aged (titres in brackets) 32 (40), 40 (40), (males); 26 (10), 33 (10), 34 (320), 37 (20), 43 (80), 44 (640), and 45 (20), (females).

Comment

The particular diseases from which our non-thyroid positive reactors were suffering (table II) are not predominantly diseases of women, and seem to bear no relation to what is known of thyroid function unless one considers that (as indeed is possible) this can be upset in any disease, past or present, with release of antigenic thyroglobulin. If this is the reason for the presence of the antibodies it carries the implication that 1 man in 10 or 1 woman in 4 in the medical beds of an Australian teaching hospital has or has had a disorder of the thyroid gland

As for their effects, one can only say from a clinical point of view that if thyroglobulin antibodies are found in the sera of as many as 18% of a large number of mixed medical patients who have not thyroid disease, then, whatever has provoked them, they are unlikely to be damaging the thyroid gland in any serious way, and it is quite possible that their role is physiological rather than pathological.

Goudie et al. (1959) tested the sera of 486 hospital patients without clinical thyroid disease. Using complement-fixation with thyrotoxic antigen, they found 6.8% positives, with some additional weak reactors. When analysed by sex and age most of those giving definite reactions were women over 50 years old. Necropsy studies showed a correlation between the presence of thyroid

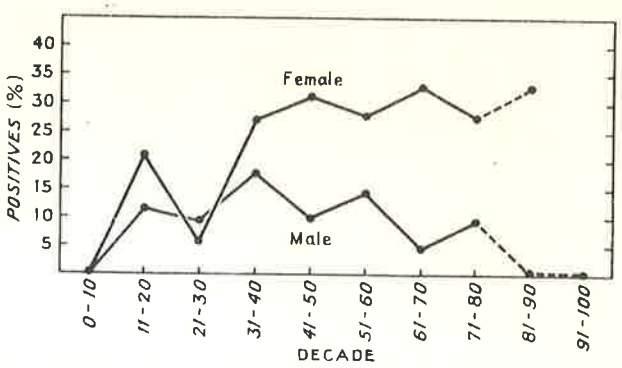

Fig. 1—Tanned-cell hæmagglutination (thyroglobulin). Non-thyroid cases. Relation to age.

The numbers of cases in each decade from which the percentages were calculated were:

	0–10	11–20	21–30	31–40	41–50	51–60	61–70	71–80	81–90	91–100
Males	5	17	22	28	39	34	37	21	6	2
Females	3	19	16	26	29	29	30	22	6	0

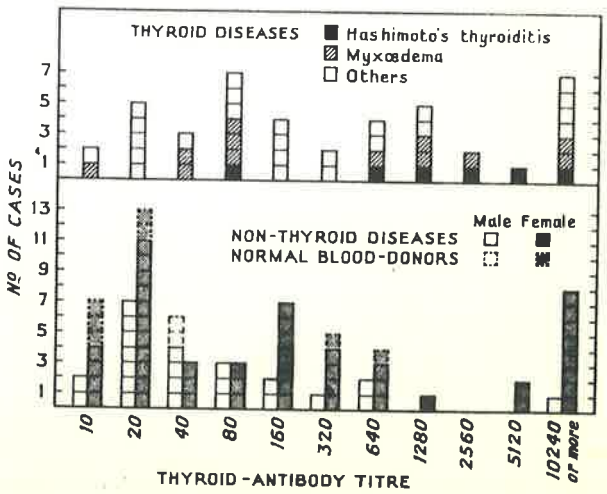

Fig. 2—Above: tanned-cell hæmagglutination (thyroglobulin) titres in 42 positives out of 69 thyroid cases tested. Below: the same in 68 positives out of 387 non-thyroid cases, and in 9 positives out of 102 healthy blood-donors.

antibody and the occurrence of mild Hashimoto-like histological changes in the gland, but this correlation was not absolute, a certain number of cases showing the changes in the gland without complement-fixing antibody in the serum. They report in the same paper that in 300 thyroid glands removed at necropsy from subjects over 40 years old the incidence of lymphocytic infiltration was 6% of 130 males and 30% of 148 females. The incidence of positives in persons over 40 in our series is similar to this (9% and 30% respectively). Possibly a finding of thyroglobulin antibodies indicates this histological condition in a person without clinical thyroid disease.

Summary

The sera of 69 cases of thyroid disease, and of 387 mixed cases without thyroid disease, were tested by the tanned-cell hæmagglutination technique for thyroglobulin antibody. Positive results were obtained in 59% of the thyroid cases and in 18% of the cases without thyroid disease.

We wish to thank Professor Robson and other consultants of the Royal Adelaide Hospital for allowing us access to their cases; Dr. Kathleen Maros for help with the sorting and recording of case-histories; Mrs. Stuart of the Walter and Eliza Hall Institute, Melbourne, for showing M. B. her tanned-cell hæmagglutination technique; and Miss N. Deering, and Miss B. Rainsford for valuable technical assistance.

One of us (E. H.) is in receipt of a grant from the National Health and Medical Research Council of Australia.

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APPENDIX 1

MATERIALS FOR TISSUE CULTURE

Collagenase: Worthington Biochemicals Corporation, Freehold, New Jersey, U.S.A.

Trypsin 1:250 crystalline: Difco Laboratories Inc., Detroit, Michigan, U.S.A.

Ficin (crude): L. Light & Co. Ltd., Colnbrook, Bucks.

Papain (crude): British Drug Houses Ltd., Poole, Dorset.

Phosphate-buffered saline with added magnesium and calcium.

(PBS Dulbecco).

Reference:- Dulbecco & Vogt (1954)

Solution A.	Grammes	
NaCl	8	
K Cl.	0.2	
Na_2HPO_4	1.15	
KH_2PO_4	0.2	
H_2O		800 ml.
Solution B		
$CaCl_2$	0.1	
H_2O		100 ml.
Solution C		
$MgCl_2\ GH_2O$	0.1	
H_2O		100 ml.

Autoclave separately at 10 lb. for 10 minutes.

Mix B and C into A when cool.

Add aqueous penicillin 100,000 units.

Parker's 199 Medium (Morton, Morgan & Parker 1950)

Glaxo Laboratories Ltd., Greenford, Middx. containing penicillin 200 units/ml. and streptomycin 100 ug./ml.

C. & M. detergent:

'Calgon' Albright & Wilson (Mfg.) Ltd., London , 40 g.

APPENDIX 1
(continued)

Sodium metasilicate 360 g., distilled water 1 gallon.

This solution is diluted one hundred times for use.

Plastic rings for culture chambers: P.T.F.E. plastic.

Richard Klinger Ltd., Sidcup, Kent.

Silicone grease: High vacuum silicone grease.

Edwards High Vacuum Ltd., Crawley, Sussex.

Preparation of apparatus.

The general requirements for tissue culture were observed. All glassware and chambers were washed by boiling in C and M. detergent solution for 20 minutes in a stainless steel bucket. Prolonged rinsing in running water was followed by washing twice in distilled water. Chambers and glassware were sterilized by dry heat (160° C. for 1 hour).

Chambers were made up using 3" x 1" microscope slides, plastic rings containing 0.8 ml. and coverslips. The adhesive was silicone grease. Chambers when assembled, with the upper surface of the ring greased, were sterilized by dry heat.

APPENDIX 2

DETECTION OF THYROID ANTIBODIES BY COONS' FLOURESCENT ANTIBODY TECHNIQUE

a. Thyroid colloid antibodies.

Method. Thyroid tissue was obtained at operation. The best tissue was obtained from patients with Graves' disease - fleshy and homogeneous tissue having even follicles when seen in section.

Tissue was collected in a polythene bag, placed on ice in a Dewar flask for transport, and cut into cubes of approximately ½" sides. These cubes were put on the side of a large test tube and immediately put into storage on solid carbon dioxide.

Five μ. sections were cut in a cryostat at -20° and processed as follows:

1. Allow cut sections to dry.
2. Fix in absolute methyl alcohol 1 hour at room temperature.
3. Allow to dry at room temperature.
4. Immerse in 70% methyl alcohol 10 seconds.
5. Rinse free of alcohol in Coons' buffer*.

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* Coons' buffer

0.02 Barbitone - H.Cl. saline pH 7.4

Sodium Barbitone	20.6 g.
Sodium Chloride	850 g.
N. H.Cl.	80.6 ml.
Water to	5 litres.

Dilute with equal volume of water for use.

Coons' mounting medium
A.R. Glycerol 9 vol.
Above buffer (undiluted) 1 vol.

APPENDIX 2
(continued)

6. Dry around section with cloth.
7. Apply serum 1 drop to section and incubate at room temperature in closed box, air humidified by moist filter papers, for 30 minutes.
8. Wash in buffer 15 minutes, with agitation.
9. Dry around section with cloth.
10. Apply small standard drop of rabbit antihuman globulin - fluorescein isothiocyanate conjugate** (The drop used in the experiments reported below was approximately 0.01 ml.) Incubate in humid box at room temperature 30 minutes.
11. Wash in buffer with agitation 15 minutes.
12. Dry slide around section and mount under coverslip with Coons' mounting medium, see below.
13. View with fluorescence microscope.

(Equipment: Zeiss ultraviolet lamp, dark ground condenser, lamp filter 1111, and Wratten 2A filters in oculars.)

b. Cytoplasmic antibodies.

Method. The method was the same as that described in (a), with the exception that unfixed thyroid tissue was used.

** Prepared in the laboratories of Dr. I. M. Roitt by the method of Riggs et al. (1958), (Am. J. Path. 34; 1081)

Part III Studies of cytotoxicity using P^{32}

STUDIES OF CYTOTOXICITY USING P^{32}

The presence of the thyroid cytotoxic factor was determined by a visual assessment of cell damage. The factor is extremely potent and active in high dilutions. Difficulty is experienced in deciding whether the factor is present only when it is present in very small amounts, or in the case of cells which are relatively insensitive to the factor. In these situations a method of quantitating cytotoxic effects is required.

No satisfactory technique for measuring the extent of damage to cells in monolayer culture could be found in the literature. Damage to cells in suspension can be measured simply by dye exclusion-techniques.

Reculture of cells surviving in monolayer, and counts of the colonies emerging, seemed impractical. No substance comparable to haemoglobin is known in cells other than erythrocytes; haemoglobin is released only when the cell is ruptured and is quantitated with the greatest ease.

The studies of the use of P^{32} were undertaken to measure cell damage objectively in a system where the observed cytotoxic effect was small, and where a substantial proportion of cells remained intact after the test procedure.

Difficulties encountered in quantitating the effects of cytotoxic sera and lymphocytes on the Wollman 16-6 rat thyroid carcinoma in monolayer culture led to an investigation of the use of P^{32} for this purpose.

The method which has been developed has general application in the detection of cell damage induced by immunological and other agents, and in the study of the kinetics of antibody action. The principle upon which the method depends is simple. P^{32} passes from cells into normal medium at a rate which is a characteristic of the particular strain of cell. Several types of toxic substance which were investigated caused an increased rate of loss of P^{32} from cells.

Data are presented to demonstrate this principle, to show the sensitivity of the technique when it is used to detect cytotoxicity, and to show the reproducibility of the results obtained with it.

MATERIALS AND METHODS

Cell cultures: Primary monolayer cultures of the 16-6 transplantable rat thyroid carcinoma (1)* were obtained using trypsin-dispersed cells. Stock cultures were subcultured by dispersing the cells with trypsin. Monolayer cultures of rat kidney cells were established using cells released by digestion of minced tissue with collagenase. Stock cultures were subcultured by dispersing the cells with trypsin. Cultures were established in Eagle's medium (L-strain modification, Difco Laboratories, Detroit, Michigan) and were maintained in Medium 199 (2). Fifteen percent of fetal calf serum was used in all cultures. Cultures of a line of the mouse L-fibroblast+ were also used. These

* Obtained from Dr. S.H.Wollman, National Institutes of Health, Bethesda, Maryland.

+ Obtained from Dr. D.J.Merchant, Department of Bacteriology, University of Michigan, Ann Arbor, Michigan.

were maintained in medium 199 containing 15% fetal calf serum. For use in tests 1 ml volumes, containing 10^5 cells in subcultures and 10^6 cells in primary cultures, were inoculated in screw-capped tissue culture tubes in a sloping position for 24 hours at 37° C. The medium was then replaced by 4 ml of the same and the tubes were incubated vertically. The medium was subsequently changed twice weekly. 2 ml of cells were inoculated into vertical tubes in later experiments, and 2 ml of medium was used throughout.

Incorporation of P^{32}: The medium in established cultures was changed for one containing 2 µc per ml of P^{32} as trisodium phosphate (Abbott Laboratories, Radio-Pharmaceuticals, Oak Ridge, Tennessee). After 24 hours at 37° C the cells were washed twice with 4 ml of 199 solution.

Use of Cells which have Incorporated P^{32}: Media containing serum, lymphocytes or substances to be tested for cytotoxicity were added in 4 ml volumes to cultures, and control tubes containing normal serum or cell suspensions were set up at the same time. Tubes were incubated at 37° C for the period of time considered necessary for the completion of the reaction or for the development of detectable cell damage.

Measurement of P^{32}: Since P^{32} is a pure beta-emitter with a maximum energy of 1.7 mev, a scintillation well-type counter was used to measure Bremsstrahlung radiation. Using a pulse-height analyser, all energies above 0.01 mev were detected. Total P^{32} counts were usually 20 times background. After measuring the total P^{32} activity the medium was poured off. The cells were washed twice by pouring in 4 ml of phosphate buffered saline (PBS) (3). The pooled

medium and washings were centrifuged at 2000 rpm for 5 minutes and a 4 ml aliquot was pipetted off for counting. The results were expressed as the percentage of the total P^{32} which had passed from the cells into the medium.

Measurement of P^{32} in Trichloroacetic Acid Precipitate of Medium: An equal volume of cold 20% trichloroacetic acid (TCA) was added to the pooled medium from replicate cultures. The precipitate was centrifuged at 2,500 rpm for 5 minutes, and washed twice by resuspending in 10% TCA and centrifuging. The washed precipitate was dissolved in 10% NaOH for counting.

Cell Counts: In an experiment in which the counts of P^{32} in washed cells were compared with the total cell counts of the corresponding cultures, cells were removed from the glass for counting by changing the medium for 199 solution containing 0.15% trypsin at pH 7.6. The tubes were agitated gently in a Dubnoff incubator at 37° C for 30 minutes. Three counts were made in a Coulter Model A counter (Coulter Electronics, Chicago, Illinois), and averaged.

RESULTS

The Rate of P^{32} Loss from Cells: Figure 1a shows the total P^{32} counts, and the proportions of these counts due to P^{32} in medium and cells, in an experiment in which replicate primary cultures of 16-6 rat thyroid carcinoma were subjected, after incubation with P^{32}, to the action of 30% normal rabbit serum #A, and to 10% NaOH, for 18 hours. Fifteen percent fetal calf serum in medium 199 was used as the control in the experiments to be described, as this combination was the best which had been found for the growth of the 16-6 and rat kidney cells.

The total P^{32} counts, and hence the P^{32} in the cells at the beginning of the test, varied greatly from tube to tube. The variation in the number of cells in the tubes undoubtedly contributed to this (see Figure 5). These data show that absolute figures of counts per minute from cells or medium cannot be used to assess the effect of a mildly cytotoxic agent, since the individual variation may be greater than the change caused by the agent.

Figure 1b illustrates, however, that the amount of P^{32} leaving the cells of the culture in 24 hours was proportional to the original P^{32} content of the cells. The percentage of the total P^{32} which had passed into the medium fell within a narrow range in replicate cultures. This relationship held over a wide range of P^{32} uptakes.

Not only did the cell-P^{32} percentages of replicate cultures in the same experiment fall within a narrow range, but also other comparable experiments carried out over the same reaction time gave results which were in the same range.

The amount of P^{32} in the medium varied with the reaction time (Table 1). Using 16-6 cells in non-toxic medium the percentage of P^{32} in the medium at 20 minutes was 8.8% ($\pm$ 2.1) (mean and standard deviation). At approximately 3 hours the mean percentage of the P^{32} in the medium fell between 21.4% and 30.4% in 11 experiments. In 3 experiments the mean P^{32} content of the medium at 18 hours was 42.8% ($\pm$ 1.6), 33.7% ($\pm$ 5.0), and 42.3% ($\pm$ 2.5).

The rate of loss of P^{32} from the mouse L-fibroblast was less than from the 16-6 cells. The P^{32} content of the medium was 8.1% ($\pm$ 1.1) and 11.5% ($\pm$ 0.8) at 3 hours in two experiments with L cells, 20.2% ($\pm$ 1.3) at 22 hours, and 30.2% ($\pm$ 2.2) at 24 hours.

Figure 2 shows the P^{32} content of the medium in experiments in which cultures of 16-6 cells and L-fibroblasts were set up simultaneously and interrupted at intervals during 24 hours. With 16-6 cells there was an initial relatively rapid release of P^{32} and a steady rate of P^{32} loss after 4 hours in normal medium. A similar rate of release of P^{32} from 16-6 cells occurred in media containing sera of other species (see Table 1). 36.0% (± 2.6) was lost into a medium containing 15% fetal calf serum in 0.9% saline in 4 hours.

The loss of P^{32} from L-fibroblasts was slower, and the initial phase of rapid loss was less pronounced (Figure 2).

Effect of Cytotoxic Factors: Addition of a cytotoxic serum caused an increase in the amount of P^{32} leaving the cells. The rat-cell toxin occurring naturally in fresh rabbit serum (4) was used in many of these experiments. Normal rabbit serum #A caused visible damage to monolayers of rat cells (increased granularity of the cytoplasm, ballooning of the cell membrane and lysis of some cells), but when added to cultures even in 30% concentration at least half of the cells survived at 24 hours. Other fresh normal rabbit sera (#B, #C) were more toxic.

The mean P^{32} content of the medium when 16-6 monolayers were incubated approximately 18 hours with 30% normal rabbit serum #A was 60.7% (± 2.6) of the total P^{32}, compared with a mean content of 33.7% (± 5.0) in control medium containing 15% fetal calf serum (Figure 1b).

Figure 3 shows the effects of adding 7.5%, 15% and 30% concentrations of fresh rabbit serum #B to groups of replicate cultures for 3 hours. The percentage of P^{32} released into the medium was proportional to the concentration of serum used. The value of the test

statistic was 14 times the value needed to declare regression significant at the 1% level.

Table 2 shows the effect of heat stable antibody in the presence of complement, after incubation at 37° C for 22 hours. Rabbit antiserum #2 to an antigen prepared from pooled Fischer rat kidney, liver and spleen, and rabbit antiserum #249 to another transplantable Wollman carcinoma (1-5A tumor) were compared with control tubes containing normal rabbit serum #C. All sera were inactivated at 56° C for 30 minutes, and were used in the test at 15% concentration in the presence of 15% of fresh guinea pig serum. The natural rabbit heterotoxin is inactivated by this treatment. Cytotoxic effects were visible in the cultures containing sera #2 and #249, but no damage was seen in the control tubes.

Results with mechlorethamine hydrochloride ("Mustargen", Merck, Sharpe and Dohme, Philadelphia, Pennsylvania) are also shown in Table 2. An inverse relationship is seen between P^{32} release and the concentration of drug in the medium in the range in which it was tested. These results clearly corresponded with the cytotoxic effects seen microscopically.

Figures 1 and 4 show the effect of incubating the cells in 10% NaOH. The medium contained 96.5% (± 0.44) and 97.9% (± 0.41) of the P^{32} in these two experiments. Microscopically there was complete destruction of the cells.

Relationship of P^{32} counts to number of cells: Figure 5 shows the correlation obtained when washed 16-6 cells were counted after their P^{32} content was measured. Substantial error in counting the cells was due to the fact that a small fraction of cells could not be

dislodged in most tubes. Nevertheless the correlation coefficient was 0.77 corresponding to a p value of less than 0.005.

Reproducibility of results: The results obtained when the experiments shown in figure 1 were repeated are shown in figure 4. The same normal rabbit serum (#A) was used on 16-6 cells in first subculture, with an incubation time of 20 hours. The P^{32} loss into medium containing 30% serum #A was 72.7% (± 2.2), compared with a loss of 42.8% (± 1.6) from cells in control cultures, the difference being 29.9%, compared with a corresponding difference of 27.0% in the first experiment. When the data of this experiment were cast into an analysis of variance, the value of the test statistic was 171 times greater than that needed in order to declare the results significant at the one per cent level.

Results obtained with consecutive experiments to test the cytotoxicity of Mustargen (Table 2) also illustrate the reproducibility of the results obtained with the technique.

Use of other cell types: In experiments using cultures of Fischer rat kidney, P^{32} uptake, normal rate of loss of P^{32} from cells, and results on exposure to rabbit serum were similar to those obtained with 16-6 cells. When a culture of rat kidney cells was incubated with 30% normal rabbit serum #B for 18 hours the medium contained 93.5% (± 2.9) of the total P^{32} compared with 46.4% (± 4.8) in the medium of the control cultures. Thirty percent of rabbit serum #B caused release of 85.4% (± 3.8) compared with the control figure of 32.9% (± 7.9) in another experiment with a primary culture of kidney cells using a reaction time of 3 hours.

L-fibroblasts lost 8.6% (± 1.1) of the P^{32} at 3 hours in normal

medium, whereas 27.5% of rabbit serum caused a loss of 25.2% (± 3.6) in this time.

Comparison with P^{32} in TCA precipitate: The TCA precipitate of the control medium did not contain measurable P^{32} in 6 experiments, but in 6 tests where cell damage was detected by an increased P^{32} loss from the cells, a proportion of this P^{32} was found in the TCA precipitate.

The P^{32} which was precipitated from the medium, expressed as a percentage of the total P^{32}, is compared with the percentage of the total P^{32} in the medium in figure 6. The coefficient of correlation is 0.89 (significant at the 1% level).

The TCA - precipitable P^{32} was also shown to be proportional to the concentration of fresh rabbit serum in the medium (figure 3).

DISCUSSION

The studies which have been reported in this paper show that P^{32} passes from cells into a normal medium at a rate which is characteristic of the type of cell, and that the loss is accelerated when cells are damaged. It is, therefore, not necessary, when comparing the effects of substances on the rate of P^{32} loss, to have identical numbers of cells in the replicate cultures, since the rate of loss is determined by measuring the ratio of P^{32} in the medium to the total P^{32} (i.e. that which was in the cells at the beginning of the test).

The rate at which P^{32} passed into the normal medium was slower with the L-fibroblast than with the cultures of rat cells which were used. The initial more rapid loss of P^{32} from the 16-6 and rat kidney cells may be due to a small amount of toxic substance in the fresh "normal" medium. However, the rate of P^{32} loss in the first 4 hours was not influenced by reducing the phosphate concentration

of the medium, or by heating the fetal calf serum used in the medium. The rate was similar in Eagle's basal medium, and medium 199 with fetal calf serum; also in media containing fresh or heated guinea pig serum, fresh rat serum, and heated rabbit serum. The rate of P^{32} loss may be a useful index of the suitability of the culture medium.

There can be little doubt that loss of P^{32} greater than that occurring in control cultures results from injury to cells. The correlation of cell numbers with counts of the P^{32} within the cells is evidence that loss of P^{32} is due at least in part to destruction of cells. Furthermore, microscopic evidence of cell damage was always seen in cultures where P^{32} loss was accelerated. However, it is possible that injury to cells may cause an increased rate of P^{32} release without killing them. P^{32} which can be precipitated from the medium by trichloroacetic acid apparently comes from within the cells as it was only detected in the medium when cells had been visibly damaged. The release of TCA - precipitable P^{32} was proportional to the total amount of P^{32} released. Also, in the case of the natural rat cytotoxin found in rabbit serum, the P^{32} release was proportional to the concentration of the toxin in the medium.

Other cytotoxic agents were tested: antisera to rat tissues containing heat-stable antibody, NaOH, and mechlorethamine hydrochloride ("Mustargen"). Each caused an increased rate of P^{32} loss from appropriate cells.

Results with nitrogen mustard show a curious effect: in the dosages which were used the cytotoxic effect varied inversely with the concentration of the drug. Further experiments are being carried out to elucidate this phenomenon. The technique should be valuable

in the study of chemotherapeutic agents.

A direct assessment of the number of cells destroyed by a cytotoxic agent may be obtained by comparing cell counts of cultures after the action of the agent, with the cell counts of identical control cultures (5). This method could not be used with 16-6 and rat kidney cultures because cells could not be dislodged completely from glass, and there was a great variation in the number of cells in replicate cultures.

Previous workers have utilized the loss of radioactive isotopes from cells as an index of cell damage. Magee, Sheek and Sagik (6) showed that C^{14} and S^{35} were released from cells in culture in the procedure of harvesting cells either with trypsin or by scraping. They were concerned only with the effects of manipulation over a period of 5-10 minutes. Levine (7) used P^{32} to study the effects of repeated washings with different solutions at short intervals on the integrity of cells in monolayer culture. He showed that the presence of serum in the medium used to wash the cells protected against an increased loss of P^{32} from the cells in the third and subsequent washes. He also showed that trypsin caused an increased loss of P^{32}, and that part of the P^{32} of media containing increased amounts of P^{32} was precipitated by trichloroacetic acid. Perlmann, Broberger and Klein (8) used the activity of C^{14} bound to protein in the medium as an index of cell damage. They have also used P^{32} in the same way (Perlmann, (9)).

The technique which has been described in this paper yields results which correspond to those obtained by measuring the acid-insoluble isotope content of the medium, and has advantages of simplicity and accuracy which accompany the performance of

multiple determinations. In addition, in the case of minimal damage to cells the release of TCA insoluble P^{32} is accompanied by the release of many times more soluble P^{32}, so that there is a greater margin between the radioactivity of the sample to be counted and the level of background radioactivity. The operations are greatly simplified by counting Bremsstrahlung radiations in a well-type scintillation counter from tubes containing monolayers and medium in the first count, and separated medium in the second, instead of measuring the beta emission from dried aliquots of lysed cells and medium in planchets.

There is need of a simple method of detecting damage to cells in monolayer culture with which objective, numerical data can be obtained. The method involving the use of P^{32} was developed for study of cytotoxicity of antisera to malignant cells, for analysis of the surface antigens of these cells, and for detection of lytic activity in vitro of lymphocytes sensitized to homologous cells. It will provide a parameter for gauging the rate of cytotoxic reactions, and may be valuable in studying the kinetics of the action of complement on cells in culture. Experiments with nitrogen mustard showed that the cytotoxic action of drugs used for cancer chemotherapy may be detected.

SUMMARY

A technique is described for detecting damage to cells in culture using P^{32}. The method is relatively simple and results obtained with it are reproducible.

The method depends on the principle that P^{32} passes from cells into a non-toxic medium at a rate which is a property of the particular type of cell. Cytotoxic agents cause an increased rate of leakage of P^{32} from cells.

The method was satisfactory with all of the cultures which were studied: the L-fibroblast, primary cultures of the 16-6 rat thyroid carcinoma and rat kidney, and subcultures of these rat cells.

Four types of cytotoxic agents were shown to cause an increased loss of P^{32} from the cells on which they were tested: the natural rat cytotoxin in rabbit serum, NaOH, antisera to rat tissues, and mechlorethamine hydrochloride ("Mustargen").

Data are presented to show that increased P^{32} loss is due to cell damage.

ACKNOWLEDGMENTS

The help of Dr. W.H. Beierwaltes, Director of the Thyroid Research Laboratory, University of Michigan, is gratefully acknowledged. Dr. D.J. Merchant of the Department of Bacteriology, University of Michigan, helped generously with technical advice, criticism and a supply of L-fibroblasts. The helpful criticisms of Dr. R.J. Porter of the School of Public Health, University of Michigan, are much appreciated. Statistical analyses were carried out by Dr. M.E. Patno of the School of Public Health of this University. The technical assistance of Mr. Arthur Smiltens was invaluable.

The Wellcome Trust, London, gave support in the form of a travel grant.

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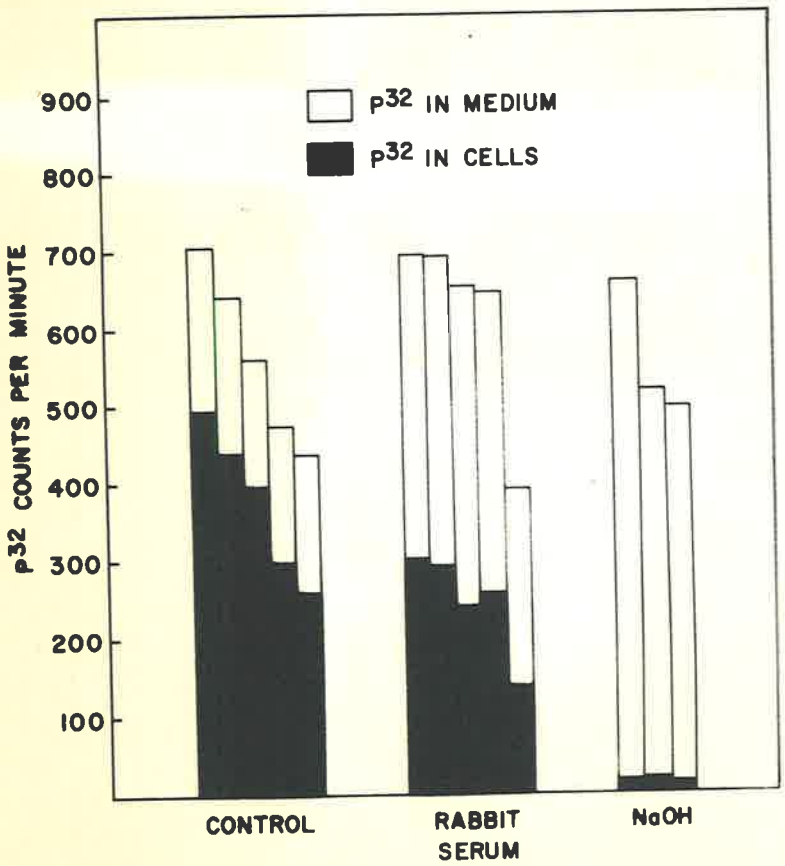
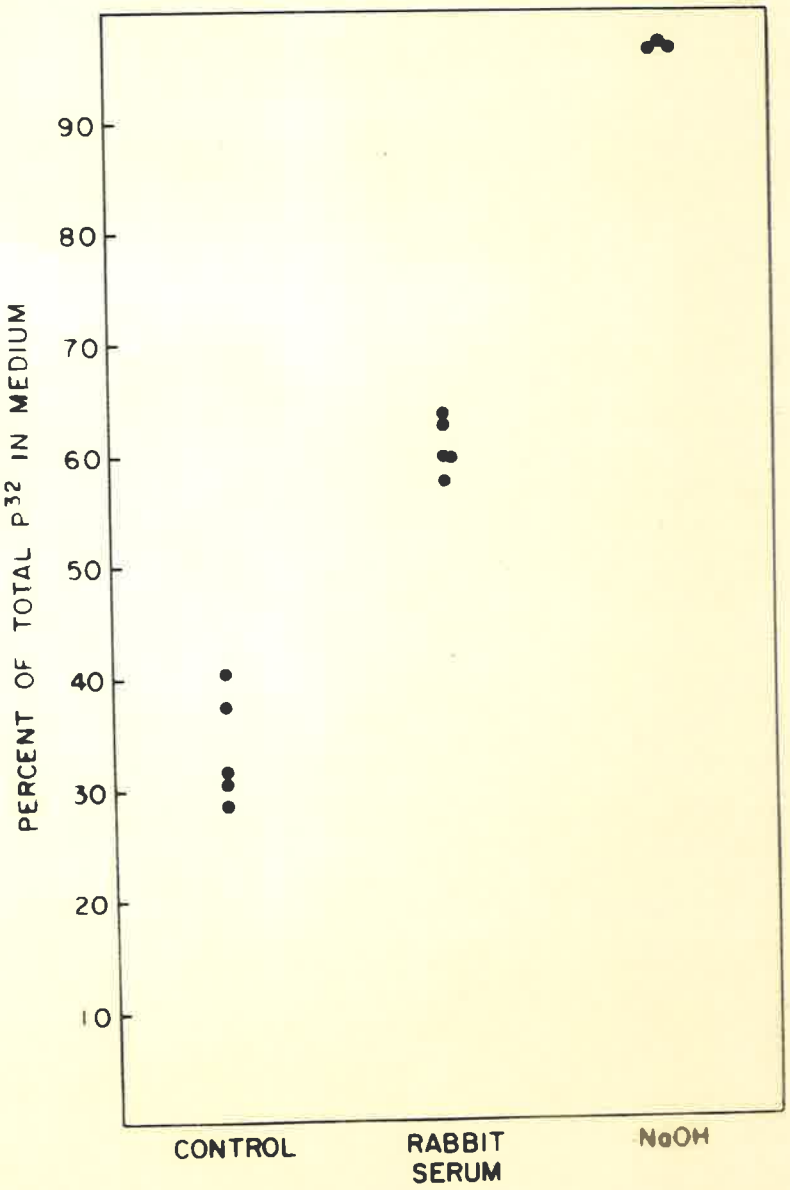
Figure 1a. The counts of P^{32} in cells (solid bars) and medium (open bars) in replicate cultures incubated in 15% fetal calf serum, 30% rabbit serum #A and 10% NaOH for 18 hours.

Figure 1b. The data expressed as the percentage of the total P^{32} which had passed into the medium at 18 hours.


p32 IN MEDIUM
p32 IN CELLS
p32 COUNTS PER MINUTE
900
800
700
600
500
400
300
200
100
CONTROL
RABBIT SERUM
NaOH


PERCENT OF TOTAL p32 IN MEDIUM
90
80
70
60
50
40
30
20
10
CONTROL
RABBIT SERUM
NaOH

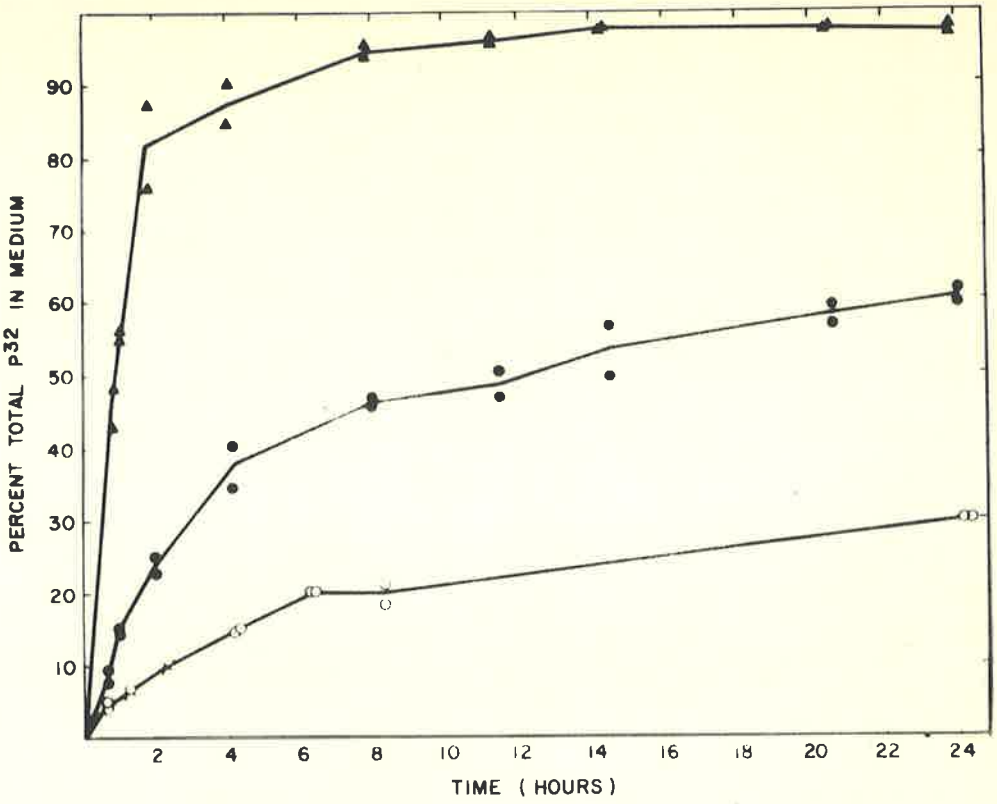


Figure 2. The percentage of the P^{32} which passed into the medium at increasing intervals of incubation of cells which had incorporated P^{32}. Open circles represent the data obtained with L-fibroblasts and solid circles the results with 16-6 cells in normal medium. The triangles plot results obtained with cultures of 16-6 cells in 10% fresh rabbit serum. Each point represents the result of one culture. The curves are drawn through the mean of each pair of observations.

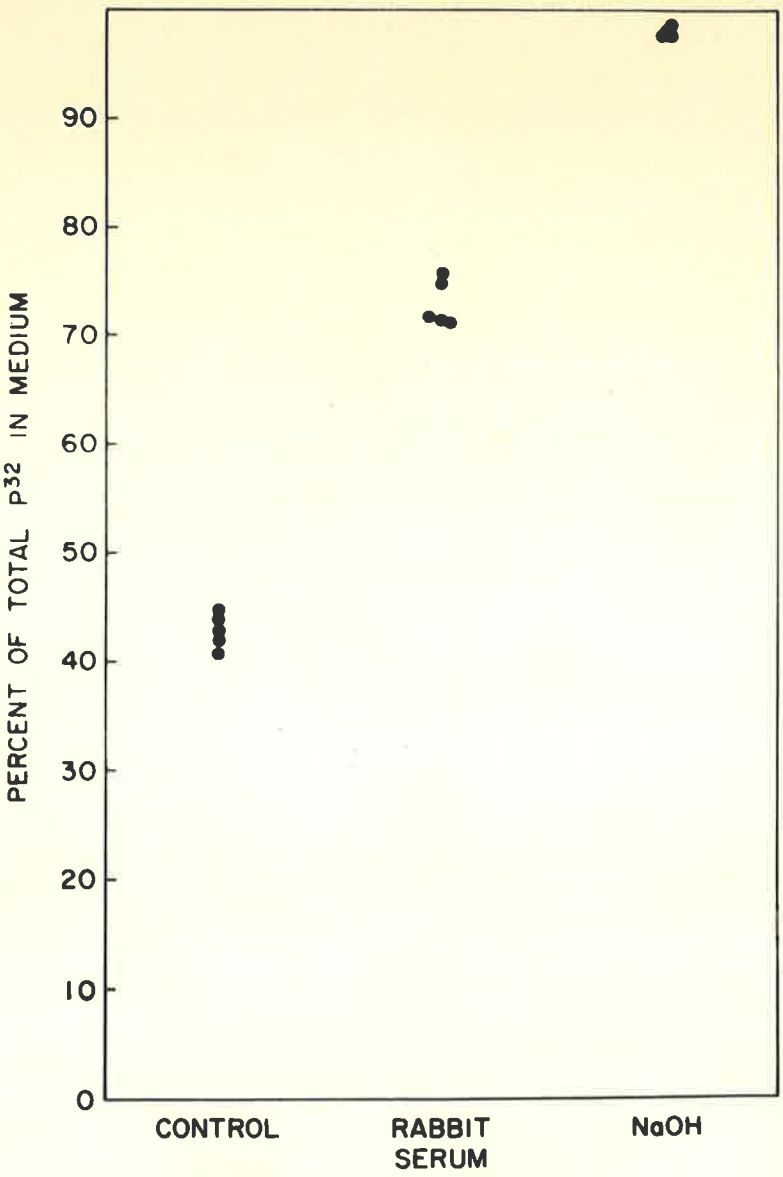


Figure 4. Results obtained when the experiments shown in figure 1 were repeated using 16-6 cells with a reaction time of 20 hours. The test medium contained 30% of rabbit serum #A.

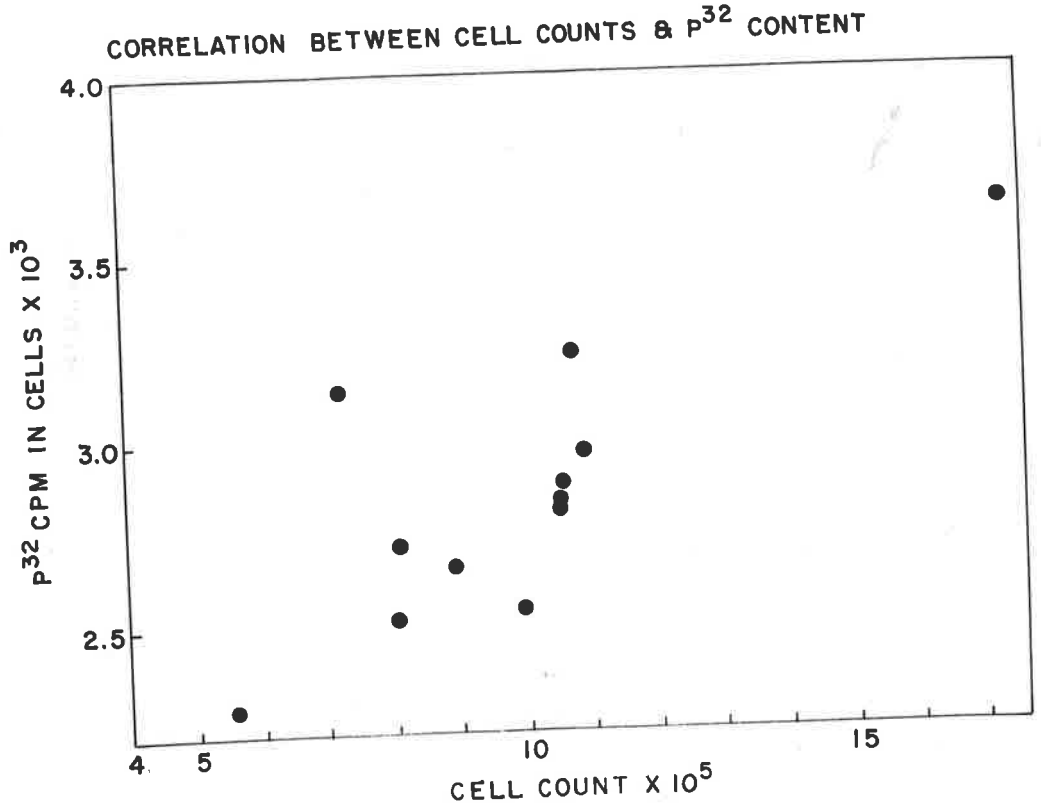


Figure 5. The P^{32} counts from washed 16-6 cells are plotted against the cell count of the corresponding culture. The cells were released from glass by treatment with trypsin and then counted in an electronic cell counter.

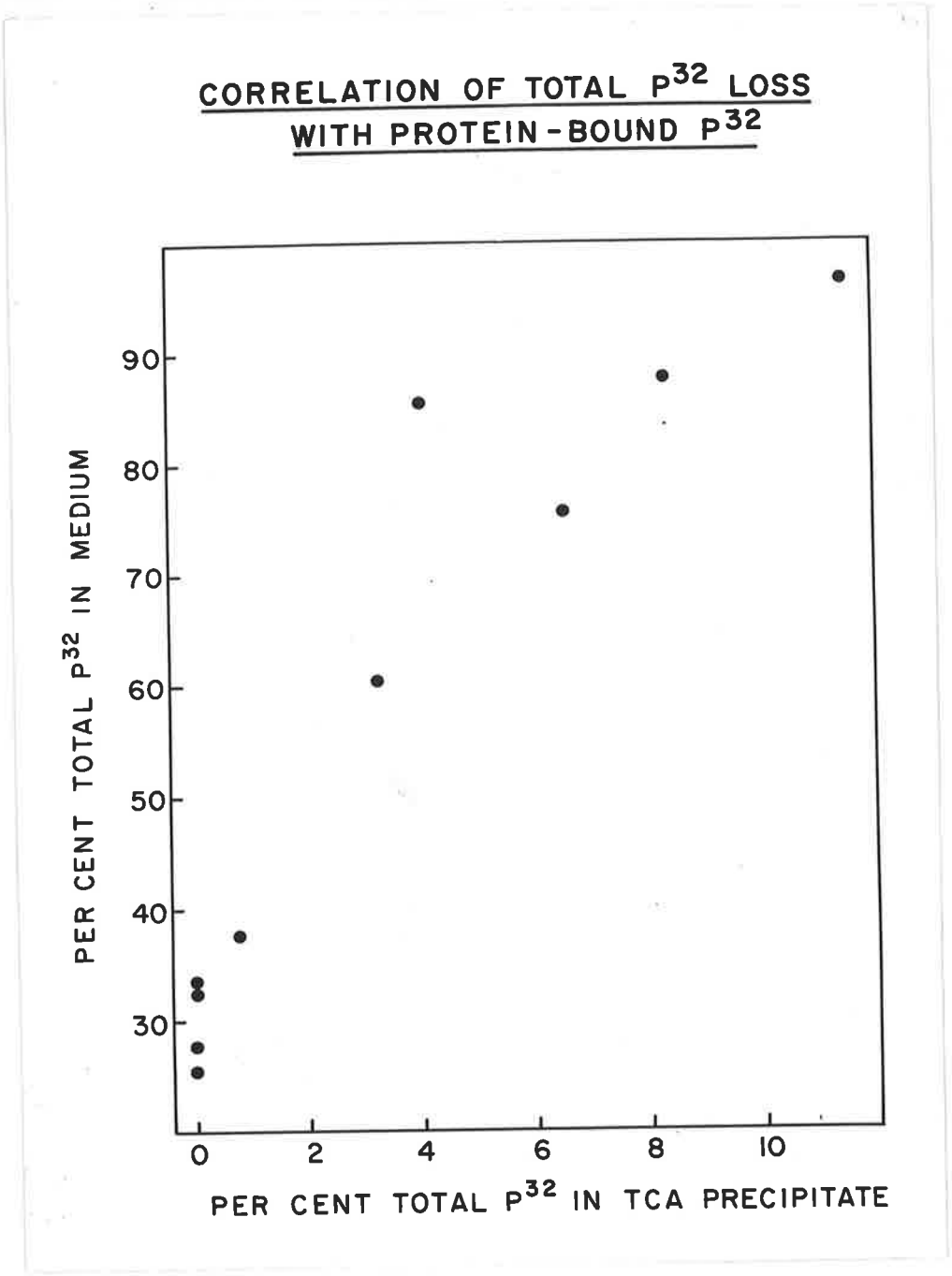


Figure 6. Mean values of the percentage of the total P^{32} in the medium for 4 or 5 replicate cultures are plotted against the P^{32} in the TCA precipitate of the pooled medium from each group of cultures, expressed as a percentage of the total P^{32}. The coefficient of correlation is 0.89.

TABLE 1

The Percentage of Total P^{32} in the Medium

Experiments with Non-Toxic Sera

Cells	Serum in Medium 199	Reaction Time	Number of Tubes	% of Total P^{32} in Medium	Standard Deviation
16.6	15% fetal calf	20 minutes	4	8.8	2.1
16.6	15% fetal calf	3 hours	3	25.4	4.5
16.6	15% fetal calf	3 hours	5	27.6	2.4
16.6	15% fetal calf	3 hours	5	30.4	2.1
16.6	15% fetal calf	3 hours	4	30.0	1.9
16.6	15% fetal calf	3 hours	4	22.4	1.1
16.6	15% heated fetal calf	3 hours	4	30.2	2.1
16.6	10% guinea pig	3 hours	4	28.2	3.1
16.6	20% guinea pig	3 hours	4	21.4	2.1
16.6	10% heated rabbit 10% guinea pig	3 hours	4	25.4	2.8
16.6	10% heated rabbit 10% guinea pig	3 hours	4	25.1	2.5
16.6	5% heated rabbit 5% guinea pig	3 hours	5	30.1	2.1
Rat Kidney	5% heated rabbit 5% guinea pig	3 hours	5	30.4	2.1
Rat Kidney	15% fetal calf	3 hours	5	32.9	7.9
16.6	15% fetal calf	18 hours	5	42.8	1.6
16.6	15% fetal calf	18 hours	5	33.7	5.0
16.6	10% normal rat	18 hours	5	42.3	2.5
Rat Kidney	15% fetal calf	18 hours	5	46.4	4.8
16.6	15% heated rabbit 15% guinea pig	22 hours	5	54.7	2.3
L-cells	15% fetal calf	3 hours	5	11.5	0.8
L-cells	1% peptone no serum	3 hours	5	8.1	1.1
L-cells	15% fetal calf	22 hours	4	20.2	1.3
L-cells	15% fetal calf	24 hours	5	30.2	2.2

TABLE 2

Effect of Cytotoxic Factors. Percentage of P^{32} in Medium.

Cell Type	Incubation Period (hours)	Toxic Agent	Percentage P^{32} in Medium Control S.D.*	Test S.D.*
16.6	22	Antiserum #2	54.7 ± 2.3	73.6 ± 2.4
		Antiserum #249	54.7 ± 2.3	76.9 ± 3.1
L-fibroblast	24	Mustargen+100 γ/ml	30.2 ± 2.9	35.1 ± 4.7
		Mustargen 50 γ/ml	30.2 ± 2.9	45.5 ± 3.3
		Mustargen 25 γ/ml	30.2 ± 2.9	58.5 ± 1.8
L-fibroblast	22	Mustargen 100 γ/ml	20.2 ± 1.3	29.7 ± 2.2
		Mustargen 50 γ/ml	20.2 ± 1.3	34.9 ± 5.1
		Mustargen 25 γ/ml	20.2 ± 1.3	45.8 ± 5.0

* Standard Deviation

\+ Mechlorethamine hydrochloride