

THE INVESTIGATION OF AUTOIMMUNE DISORDERS

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Introduction

During the last few years our knowledge of autoimmune disease has increased considerably, and the techniques for detecting such disease have been improved and made simpler. The laboratory tests to detect humoral antibodies in patients suffering from an autoimmune disorder have become routine in a number of hospitals and the use of techniques, and, in particular immunofluorescence techniques, have assumed greater importance.

The purpose of the present communication is to outline some of the available immunopathological techniques and to illustrate how they are applied in the day to day investigation of patients in an acute general hospital.

Immunopathological Techniques

A) *Immunofluorescent methods*

The indirect immunofluorescence technique — also called the “sandwich” method — is used in most cases (Nairn 1969). Normal tissue is snap-frozen in a liquid nitrogen-isopentane mixture (temperature -180°C) and stored for future use at -70°C . When required, frozen sections are cut and the patient's serum layered on top of the section. (*Fig. 1*). Antibodies present in the serum will combine with antigenic sites in the tissue section. Excess serum is then washed off, and a fluorescense-conjugated rabbit anti-human globulin is applied to the section

— this last layer will attach to any globulin remaining on the section, thereby localising any antigen-antibody reaction.

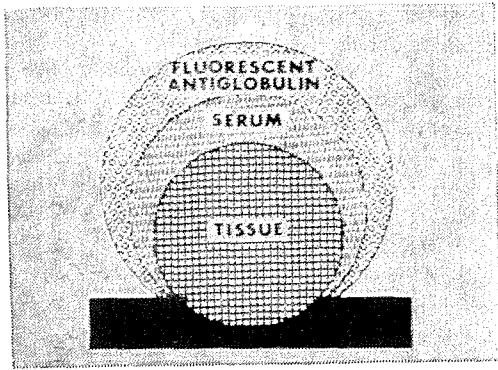


Fig. 1

Using this method a number of tests have been devised to detect antibodies in human sera. Antinuclear antibodies (*Fig. 2*) are best detected using rat liver sections.

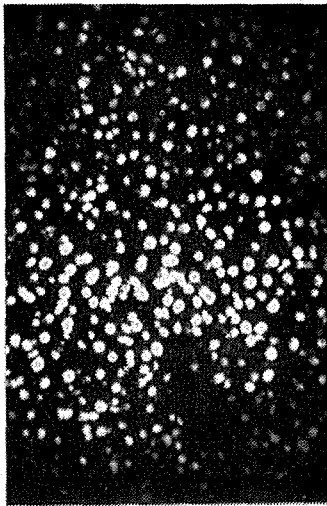


Fig. 2

Such antibodies are present in about 100% of patients suffering from lupus erythematosus. Antibodies to gastric parietal cells can likewise be demonstrated in the sera of 90% of patients suffering from pernicious anaemia. (McKay, 1969) (*Fig. 3*). In autoimmune disorders affecting the thyroid gland, two distinct antibodies may be detected by immunofluorescence. First-

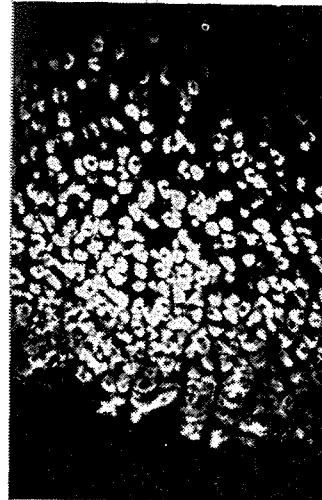


Fig. 3

ly a cytoplasmic antibody reacting with the cytoplasm of cells lining the vesicles (*Fig. 4*), and secondly, an antibody reacting with thyroglobulin (*Fig. 5*). Antithyroglobulin antibodies are present in 90% of patients with Hashimoto's disease and



Fig. 4

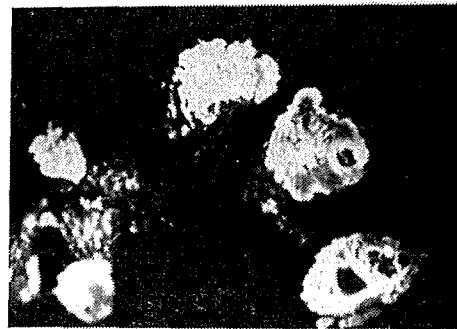


Fig. 5

anticytoplasmic antibodies are present in about 70% of patients with thyrotoxicosis.

Other immunofluorescence tests that yield useful information are:

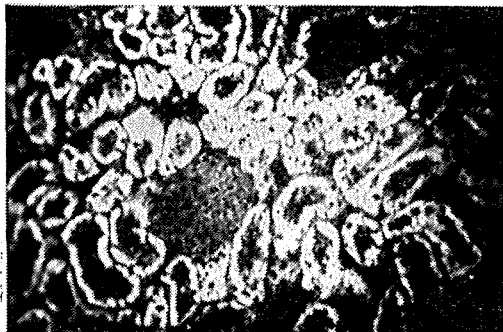


Fig. 6

a) Antimitochondrial antibodies (*Fig. 6*). These antibodies, best detected in kidney tubule cells are present in about 79-94% of patients with primary biliary cirrhosis, and since such antibodies are not present in patients with extrahepatic jaundice, their presence is useful in the differential diagnosis of these two conditions. (*Brit. Med. J. 1970, Doniach 1968*).

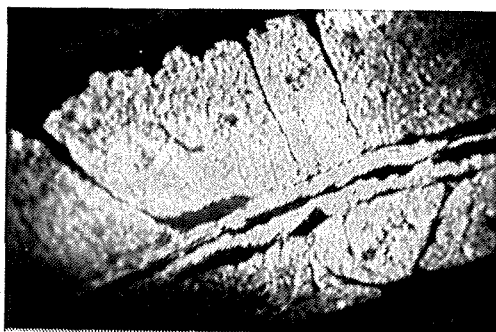


Fig. 7

b) Anti smooth muscle antibodies (*Fig. 7*). These are present in cases of lupoid hepatitis.

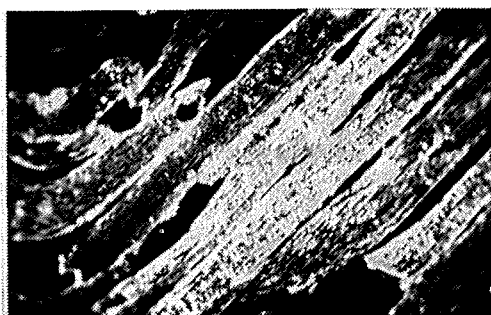


Fig. 8

c) Anti striated muscle antibodies (*Fig. 8*) are present in cases of myasthenia gravis, about 30% of which show the typical band fluorescence.

d) Anti adrenal cortex antibodies are present in about 50% of patients with Addison's disease.

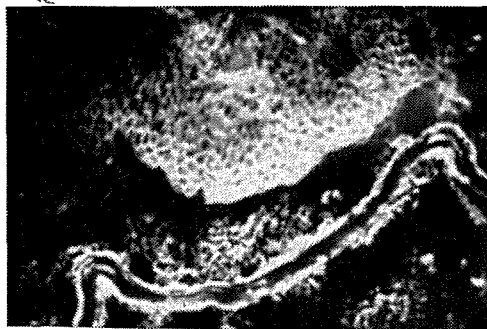


Fig. 9

e) In skin disorders, antibodies against the intercellular area and cell membranes of squamous epithelium are present in pemphigus vulgaris (*Fig. 9*), while anti-basement membrane zone antibodies are present in Bullous pemphigoid (*Fig. 10*). So characteristic are these changes that the presence of such antibodies is virtually diagnostic. (*Muller and Sutherland, 1970*).

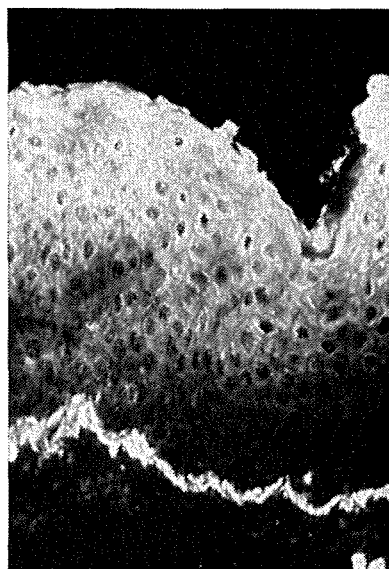


Fig. 10

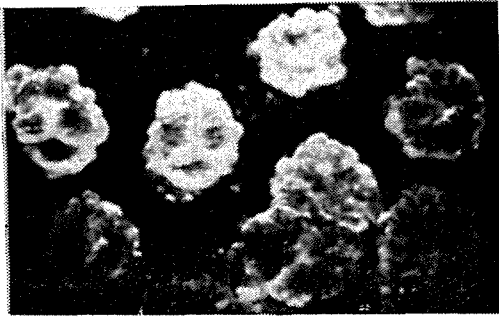


Fig. 11

f) In ulcerative colitis, anticolon mucous antibodies can be detected (Fig. 11). (McGiven *et al.*, 1967).

g) Using specific antisera to immunoglobulin IgG, IgM, IgA as well as anti-

fibrin, anti complement etc., direct visualisation of the exact nature of antigen antibody complex can be achieved. (This is an example of the direct immunofluorescent technique, as opposed to the above test where the indirect test is used).

B) Tanned Red Cell Haemagglutination Technique

Tanned sheep red cells have the power of combining with a number of antigens (Herbert, 1967). Thyroglobulin is such an antigen that can be readily attached to sheep red cells to produce a very useful reagent for the detection of antithyroglobulin antibodies. Briefly, thyroglobulin-coated cells (supplied by "Wellcome") are added to a series of tubes containing se-

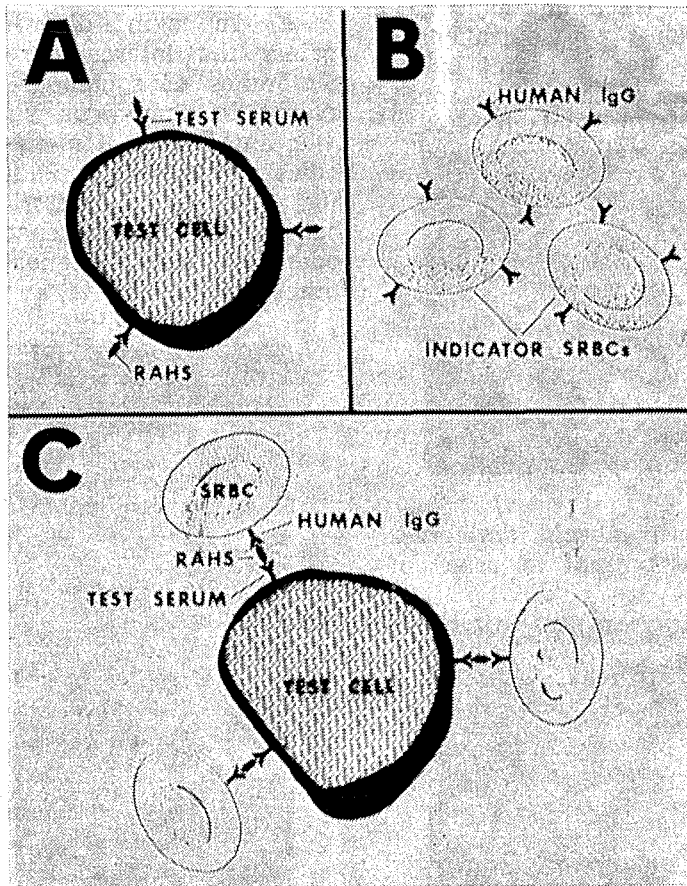


Fig. 12

rial dilutions of the patient's serum. Agglutination of the red cells occurs in those tubes containing sufficient antibody. In Hashimoto's disease, this test is positive in 90% of cases (McKay, 1969).

C) Latex Agglutination

In rheumatoid arthritis, the latex agglutination test is used to detect rheumatoid factor. Latex particles coated with human γ globulin are supplied in kit form (Hyland). When one drop of test serum (diluted 1/20) is added to 1 drop of latex-globulin reagent, macroscopic clumping occurs.

Other Tests Not Used Routinely

A) Mixed Antiglobulin Test (Coombs and Gell, 1968)

This is a very sensitive test for detecting very small amounts of circulating antibodies to cellular constituents, such as peripheral blood lymphocytes, tumour cells, etc. In principle, (Fig. 12), lymphocytes or other cells to be tested are washed free of serum and the test serum is then added. After a suitable interval, the cells are washed again and antihuman serum (e.g. rabbit antihuman globulin) is added so that the cells are coated with a layer of human serum, and then with a second layer of rabbit anti-human globulin (Fig. 12A). As an indicator system, sheep red cells coated with human globulin are used (Fig. 12B). When mixed together, typical

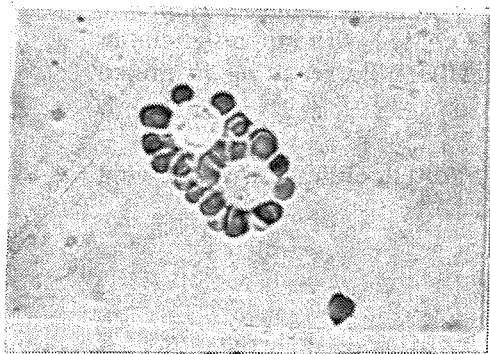


Fig. 13

rosettes are formed consisting of a central lymphocyte surrounded by sheep red cells (Fig. 12C, Fig. 13). This technique can be used to demonstrate specific antibodies to cell membranes such as antibodies to tumour cells (Mori and Coombs, 1969).

B) Membrane Immunofluorescence

In routine immunofluorescence tests, the tissue is frozen — a process which kills cells — and then serum is layered on, so that it comes in contact with the intracellular constituents, e.g. cytoplasm, nuclei etc. The technique of membrane immunofluorescence enables the detection of antigens present on the membrane of cells, and in this context it is well to remember that the cell membrane may well be a very important antigenic constituent in a number of immunological situations.

Theoretically, the technique of membrane immunofluorescence is quite simple. The cell suspension to be tested (e.g. tumour cells) is first washed, and then the test serum is added. After half an hour, the cells are again washed and fluorescencconjugated antihuman globulin added. A drop of cells is then examined by fluorescent microscopy. When the reaction is positive, the cell surface shows a ring of bright fluorescence.

The main value of this technique is to localise antigens situated on the cell surface, such as tumour specific transplantation antigens, HL-A antigens, etc.

The Routine Investigation of Patients Suffering from Autoimmune Disease

The rest of this communication will deal with the results obtained from tests performed on 1000 consecutive patients suffering from a number of unrelated disorders. It is to be emphasised that these results do not in any way represent a survey of the incidence of the various diseases. Rather it is hoped that these results will illustrate a number of facets common to autoimmune disease in general.

As seen in Table 1, out of 1000 patients tested, 481 had one or more positive tests. The very high incidence illustrates the selection of cases referred for

LEGENDS TO FIGURES:

- Fig. 1.** Diagrammatic representation of the indirect immunofluorescence test. (See text).
- Fig. 2.** Anti nuclear antibodies. Liver sections treated with anti ANF sera show bright staining of cell nuclei.
- Fig. 3.** Anti gastric parietal cell antibodies. Sections of stomach fundus to show positive parietal cell staining.
- Fig. 4.** Anti thyroid cytoplasmic antibodies to show staining of cytoplasm in human thyroid.
- Fig. 5.** Anti thyroglobulin. Typical flocculent staining of colloid inside thyroid vesicles.
- Fig. 6.** Anti mitochondrial antibodies. Kidney sections showing cells lining the tubules staining positively. The glomeruli are negative.
- Fig. 7.** Anti smooth muscle antibodies.
- Fig. 8.** Anti striated muscle antibodies.
- Fig. 9.** Anti bodies to intercellular area from a case of pemphigus vulgaris (courtesy of Dr. H. K. Muller).
- Fig. 10.** Anti basement membrane zone staining in Bullous pemphigoid (courtesy of Dr. H. K. Muller).
- Fig. 11.** Anti colon mucus antibodies in ulcerative colitis.
- Fig. 12.** Principle of the mixed antiglobulin test:
A: Test cell coated with test serum and anti human serum.
B: Indicator Sheep Red Blood Cells coated with human globulin.
C: Mixing of test cells with indicator cells resulting in mixed agglutination.
- Fig. 13.** Rosette formation in the Mixed Antiglobulin Test, showing lymphocytes surrounded by red cells.

TABLE 1
Incidence and sex ratio of patients
having one or more positive
immunological tests

		M : F Ratio
Number of patients tested	1000	1 : 1.5
Number of patients with positive tests	481	1 : 1.9

immunological studies. It is to be noted that the sex ratio of patients having a positive immunological test is heavily loaded towards females in the ratio of 1 : 1.9 (compared to 1 : 1.5 for the patients in general).

TABLE 2
Incidence of individual positive results

Test	Number positive
Anti nuclear factor (ANF)	82
Anti gastric parietal cell (AGPC)	260
Anti thyroid cytoplasm (Cyto)	130
Anti thyroid colloid (Coll)	32
Anti mitochondrial (Mito)	13
Anti smooth muscle (S M)	30
Rheumatoid Factor (R.A.)	92
Tanned Red cell Agglutination (TRC)	78

Table 2 shows the incidence of the individual positive tests. Antigastric parietal cell antibodies were the commonest ones encountered (26%). Antithyroid tests (including anti-cytoplasmic, colloid, and tanned red cell tests) were positive in 3.2–13.0% of the patients, depending on the test used. Antinuclear factor was evident in 8.2% of patients.

TABLE 3
Patients having "single" or "multi"
system involvement *

Number of patients having one positive test	298
Number of patients having two positive tests	136
Number of patients having three positive tests	47

(* Anti thyroid cytoplasmic and anti thyroid colloid antibody and tanned red cell agglutination represent one single system involvement, and hence were counted as one test for the purpose of this table.)

In many cases there was more than one antibody present in the serum. Table 3 shows that 136 patients had two positive tests. (For this purpose, the three different antithyroid tests, viz, anti cytoplasmic, anti colloid and tanned red cell agglutination, were considered as one test). 47 patients had antibodies reacting with three different systems, e.g. antigastric, anti thyroid as well as anti nuclear. This relationship is further analysed in Table 4 which illustrates the frequency of association of antibodies in autoimmune disorders. For example anti parietal cell antibody is found associated with anti thyroid cytoplasmic antibody in 51 patients, and with RA test in 33 patients. Similarly, antithyroid cytoplasmic antibody is found in 15 patients having anti nuclear factor, in 24 patients having anti colloid, in 51 patients having a positive TRC, as well as in 15 patients having a positive RA.

TABLE 4
The frequency of association
of positive tests

	ANF	AGPC	CYTO	Coll.	Mito	S.m.	RA	TRC
ANF	—	17	15	2	—	2	19	12
AGPC	17	—	51	13	9	7	33	34
Cyto	15	51	—	24	4	3	15	51
Coll	2	13	24	—	1	1	5	23
RA	19	33	15	5	3	1	—	10
TRC	12	34	51	23	2	4	10	—

To be noted also the high incidence of anti gastric parietal cell positive sera in RA positive patients. RA tests were not done routinely on all patients, but there was no reason to suspect that AGPC positive patients were selected for RA tests. Tanned red cell tests were done only on those patients with a positive anti cytoplasmic or anti colloid antibody. This explains the positive correlation of immunofluorescent tests for thyroid disease and a positive TRC: Out of 78 patients with positive TRC test, 51 were associated

with a positive anti thyroid cytoplasmic antibody, and 23 with anti thyroid colloid antibody. The association of positive TRC and anti AGPC however represents the association of autoimmune disease affecting both thyroid and gastric cells.

Although the data presented above are selected and not meant to indicate the true incidence of autoimmune disease, nevertheless several important conclusions can be drawn from this study:

1) The importance of performing immunological tests on patients in a general hospital population.

2) The greater incidence of autoimmune disease in females.

3) The importance of testing all patients with a battery of tests and not just with a single test. In many instances it can be shown that the patient serum was negative for a suspected lesion, e.g. ANF, but positive for AGPC or antithyroid antibodies.

4) It has further been shown that in certain instances which are not uncommon, a "multisystem" autoimmune disorder is present associated with a whole host of abnormal antibodies.

5) Finally, in drawing any conclusions from immunological tests of this nature it is important to keep in mind the tendency for antibodies to appear with advancing age (Whittingham *et al.*, 1969). Apart from the fascinating correlation between the process of ageing and autoimmune disease which this observation entails, it is essential to keep in mind the fact that the presence of antibody is not necessarily causing the disease, but may be a secondary accompaniment of tissue breakdown and destruction. It has been shown, for example, that destruction of

peripheral blood cells by irradiation is followed by antileucocyte antibodies in the serum (Suzuki, 1969), and that the level of antibodies to Burkitt Lymphoma cells is greater after chemotherapy, which presumably involves massive destruction of tissue and cells (Yata *et al.*, 1970). It is conceivable that some similar or related process is occurring in the aged population.

In all cases, however, a careful assessment of the immunological status of patients suffering from a variety of (usually chronic) disorders, using both humoral antibody techniques described above, as well as techniques that measure the cellular immunological potential, are essential in the proper evaluation of the immunological status of patients.

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