Studies on Autoimmune-hematologic Disorders by Means of Fluorescent Antibody Methods

Immunological Mechanism of Autoimmune hemolytic Anemia and Relationship between Autoimmune Antibody and Megakaryocytes in I.T.P.

Tadashi OFUJI

*Department of Internal Medicine Okayama University Medical School
(Director Prof. Kiyoshi Hiraki)*

Introduction

Scrutiny of antibody-producing cells in autoimmune hemolytic anemia is considered to be a very important problem that will throw light on the mechanism of autoimmunity. Experimental approach to the relation between anti-thrombocytic autoimmune antibody and bone marrow megakaryocyte in I. T. P. is also an important problem concerning the pathogenesis of I. T. P. On these problems, however, no satisfactory investigations have been performed.

The purpose of this paper is to present a study on these problems by means of fluorescent antibody methods.

A. Scrutiny of antibody-producing cells in autoimmune hemolytic anemias—a confirmation of new theory of autoimmunity of Burnet and Dameshek. Although autoimmunity was understood to be due to abnormal state of the part of antigens, Burnet¹⁾ explained autoimmunity from the part of antibody producing tissues on the basis of his famous clonal selection theory proposed in 1959. In 1959 Dameshek^{2,3)} also tried to explain autoimmunity by abnormality of antibody-producing tissues in consideration of Madawar's concept of immune tolerance, he supposed that when certain pathologic changes arose in tissues of antibody formation, they might fail to identify "self" tissue and react to it as to "non-self". Furthermore, he thought that in that circumstance, antibody-producing tissues stood in the same situation as that of the tissues of antibody formation homografted from other animals in runt disease. The present study was conducted in the aim of demonstration of autoimmune antibody-producing tissues by fluorescent antibody methods of Coons^{5,6)} during the author's stay at Dr. Dameshek's laboratory in Boston. The following results seem to prove the working hypothesis of Dr. Dameshek in some extent⁷⁾.

Materials and methods

Biopsied tissues of the lymph node, bone marrow and spleen of four patients with autoimmune hemolytic anemia including a case associated with chronic lymphocytic leukemia were used as materials. Lymph node was cut by the

cryostat to give 2μ thick frozen sections, and bone marrow and spleen were used as imprinted preparations.

Three fluorescent antibody methods were used: (1) Direct method with fluorescein isothiocyanate-conjugated rabbit anti-human globulin serum was used. (Fig. 1) (2) Direct method with fluorescein-conjugated rabbit anti-serum prepared against eluates of patient's red blood cells was used. (Fig. 1,3) (3) In indirect method, first rabbit anti-eluate serum and secondly fluorescein-conjugated sheep anti-rabbit globulin serum were used. (Fig. 2).

Control specimens included lymph node and spleen of patients without blood disorders and normal human bone marrow.

Immuno-Histological Techniques.

I. Direct Method

Fig. 1 The Site of Antibody Formation in Auto-Immune Hematological Disease.

Immuno-Histological Techniques.

II. Indirect Method

Fig. 2 The Site of Antibody Formation in Auto-Immune Hematological Disease.

Fig. 3 The Site of Antibody Formation in Auto-Immune Hematological Disease

Experimental results

(A) Case of autoimmune hemolytic anemia

Biopsied materials of spleen and bone marrow obtained from three patients with this condition were comparatively examined with Giemsa-stained control specimens. In each case clear specific fluorescence was observed in the cytoplasm of plasm cells, while no specific fluorescence was seen in controls. (Tab. 1, Plates 1,2)

(B) Chronic lymphocytic leukemia complicated by autoimmune hemolytic anemia.

Table 1. Fluorescent Antibody Studies in Autoimmune Hemolytic Anemia

Disease	Tissues Studied	Results
"Idiopathic" Auto-Immune Hemolytic Anemia	Bone Marrow	Fluorescent
	Spleen Aspirate	Plasma Cells
Chronic Lymphocytic Leukemia + Auto-Immune Hemolytic Anemia	Lymph Node	Fluorescent
	Bone Marrow	Leukemic
	Spleen	Lymphocytes
No. Immunological Disease (Controls)	Bone Marrow	No Fluorescent Cells
	Spleen	
	Lymph Node	

Study of lymph node, spleen, and bone marrow from a patient with chronic lymphocytic leukemia complicated by autoimmune hemolytic anemia showed a brilliant fluorescence in the majority of leukemic lymphocytes, while control specimens from normal tissues were negative. (Tab. 1, Plate 3.)

Comment

These studies indicate that the site of antibody formation in autoimmune hemolytic anemia is the plasma cell, while the leukemic lymphocytes produce the abnormal antibodies found in chronic lymphocytic leukemia complicated by autoimmune hemolytic anemia. These observations will support the hypothesis of Dameshek that "autoimmune antibodies are derived from the abnormal state on the part of antibody-producing individuals", and furthermore will provide an experimental basis for "a new theory of autoimmune mechanism" by Burnet and Dameshek.

B. Search for the cells producing autoimmune antibodies in I.T.P. and relationship between the antibodies and bone marrow megakaryocytes.

According to Evans⁸⁹⁹ and Dameshek¹⁰⁰, I.T.P., particularly its acute form is due to anti-platelet autoimmune antibodies. However, experimental evidence is lacking to demonstrate cells which produce such antibodies, and no cytological study dealing with the relationship between the antibodies and megakaryocytes has been attempted. The fluorescent antibody methods were employed to elucidate some of these problems in the following experiment.

Materials and methods

Bone marrow smears obtained from three patients with I.T.P. were studied. Two general methods were used to stain the tissues. In the direct method fluorescein-conjugated rabbit anti-human globulin serum was used to stain the bone marrow smears. In the indirect method bone marrow smears from normal persons were first reacted with serum from the patients. After this procedure, the smears were stained with fluorescein-conjugated rabbit anti-human globulin serum. As controls, for the former method, bone marrow smears from normal persons were used, and for the latter, normal serum and serum from the patients antibodies of which were removed by absorption with normal platelets were used. (Fig. 4)

Experimental results

The direct method demonstrated a brilliant fluorescence in almost all the plasma cells and in many of the megakaryocytes of bone marrow smears from the three patients. (Tab. 2, Plate 4,5) In the indirect method only the megakaryocytes but not the plasma cells from normal bone marrow fluoresced brilliantly. All the control specimens were negative. (Tab. 2, Plate 6.)

Comment

From the fact that only the plasma cells from the patients showed the

Fig. 4 Fluorescent Antibody Studies in ITP

fluorescein-labeled antibodies, it has become clear that the plasma cells are responsible for the formation of the autoimmune antibodies in I.T.P. In addition, staining of the megakaryocytes by both methods has made it possible to label the antibodies in the platelets which are being separated or have been just separated from the megakaryocytes. It is apparent, therefore, that the antibodies exert a deleterious effect not only upon the circulating platelets but also upon the platelets during and immediately following separation from the megakaryocytes. When serum from the patients with I.T.P. antibodies of which were eliminated by absorption with normal platelets was used, the brilliant fluorescence was no longer observed. This confirms that the platelets themselves are acting as antigens.

The inability of platelet formation of megakaryocytes in I.T.P. has been well established by our clinical tissue culture method. The motility of megakaryocytes and ability to separate off platelets from their tentacles are known to be in a parallel relationship. In I.T.P. there is deficiency of C-type megakaryocytes which actively separate off the platelets. The etiology of I.T.P.

Plate 1. Spleen of a patient with autoimmune hemolytic anemia.....Fluorescence of plasma cells (direct method)

Plate 2. Normal bone marrow.....Control specimen for plate 1) (direct method)

Plate 3. Lymph node of a patient with autoimmune hemolytic anemia complicating chronic lymphocytic leukemia.....Fluorescence of leukemic lymphocytes (Indirect method)

Plate 4. Bone marrow of a patient with I. T. P. Fluorescence of plasma cells (direct method)

Plate 5. Bone marrow of a patient with I. T. P. Fluorescence of megakaryocyte (direct method)

Plate 6. Normal bone marrow Fluorescence of megakaryocyte (indirect methodusing serum of a patient with I. T. P.)

Table 2. Fluorescent Antibody Studies in ITP

Source of Tissue	Reacting Serum	Platelet Absorption	Fluorescing Cell
Patient's Marrow	Conjugated Rabbit Anti-Human Globulin	No.	Plasma Cell + Megakaryocyte
Normal Marrow	Patient's Serum Followed By Conjugated Rabbit Anti-Human Globulin	No.	Megakaryocyte
Normal Marrow	Patient's Serum Followed By Conjugated Rabbit Anti-Human Globulin	Yes	None

Interpretation The Anti-Platelet Antibody of ITP is Produced by Plasma Cells

appears to lie, therefore, in the absence of the plateletforming, active C-type megakaryocytes in the bone marrow. The present experiment has provided evidence to answer this question by revealing that in I.T.P. the anti-platelet antibodies arrest the complete separation of the platelets from the megakaryocytes by acting upon the platelets during and immediately following their genesis from the megakaryocytes.

Conclusion

On the basis of the present study, on autoimmune disorders by means of fluorescent antibody methods, the following conclusions were obtained.

1. The antibody-producing cells in idiopathic autoimmune hemolytic anemia are plasma cells.
2. The cells of antibody formation in autoimmune hemolytic anemia developed in chronic lymphocytic leukemia are leukemic lymphocytes. This provides an experimental confirmation for Dameshek's autoimmune theory of pathologic state of antibody-forming tissues.
3. The site of antibody formation in I.T.P. is the plasma cell.
4. The antigens against the antibodies in I.T.P. are platelets.

The antibodies act not only upon platelets in the peripheral blood, but also the platelets in their stage of genesis in megakaryocytes and arrest platelet separation of megakaryocyte.

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Discussion

Dr. Tanaka

What is the reason why you confirmed γ -globulin in plasma cells and lymphocytes as auto-antibody ?

Is non-specific reaction of human megakaryocytes as prominent as in those of experimental animals ?

Dr. Ohfuji

All present cases auto-immune hemolytic anemia showed positive Coon's reaction and increasing of plasma cells in which specific fluorescence was observed. Normal human megakaryocytes showed slight nonspecific fluorescence which was apparently different from specific one.

Dr. Kawamura

1) The direct, indirect and complement methods do not give characteristically different results with using highly immune sera.

2) It seems to be further necessary to investigate the purification and character of labelled antibody.

3) The control staining seems to be incomplete.

Immunohistochemical Studies on Malignant Neoplasms by Means of Fluorescent Antibody Methods

—Localization of tumor viruses in mice with mammary cancer or leukemia and site of antibody production in immune rabbit.—

Kiyoshi HIRAKI, Takashi KOTSUKA and Masanori KAHARA

Department of Internal Medicine, Okayama University Medical School

Recently, it became clear that certain mouse tumors were induced by viruses and the viruses were morphologically demonstrated by electron microscopy. The fluorescent antibody methods²⁾ have been utilized to pursue the localization of viral antigens. Many studies in this field have been performed by investigators: namely observations on viral antigen of Rous sarcoma by Mellors et al¹⁰⁾, Malmgren et al⁸⁾, Vogt et al¹⁴⁾ and Noyes¹³⁾, investigations on Shope papilloma by Noyes¹²⁾ and on Polyoma by Malmgren⁹⁾. In Japan, Chicken erythroblastosis virus was investigated by Iwakata⁶⁾ and mouse leukemia virus by Ichikawa et al⁵⁾ as studied with fluorescent antibody method. The purpose of this communication is to present a study on localization of viral antigens in C3H strain mouse mammary cancer and C 58 and AKR strain mouse leukemias, and an observation on the site of antibody production in hyperimmune rabbit.