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HUMAN ANTIBODIES TO VASCULAR ENDOTHELIUM

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SUMMARY

Certain human sera were found to produce specific staining of vascular endothelium by the immunofluorescent technique. The antibody nature of this reaction was confirmed by using fluorescein-conjugated antisera specific for human immunoglobulins and the component of complement, and by physicochemical characterization of isolated immunoglobulins giving this reaction. This activity was present in sera from patients with a wide variety of diseases (17·8 %). The highest incidence was found in chronic pulmonary tuberculosis (26·6 %). An incidence of 14 % was found in presumably normal blood donors. The stimulus for the production of these antibodies is unknown. The antigen is fairly widely distributed among different species, since tissues from a variety of animals could be used as substrate in the reaction. Experiments have shown that neither the classic Forssman antigen nor ABO blood groups are involved. The possible role of these antibodies in human disease remains to be elucidated. The finding of anti-endothelial activity in two recipients of renal transplants may be significant.

INTRODUCTION

In the course of antinuclear factor testing by immunofluorescence using mouse kidney sections as tissue substrate, a linear type of staining pattern was noted, in which specific fluorescence was seen outlining the peripheral border of the renal tubules and the glomerular tufts. Since most of the serum samples being studied came from patients suspected of having some sort of connective tissue disease, early indications were that this pattern frequently occurred with sera from patients with polymyositis or dermatomyositis. In subsequent studies this activity was found in a variety of disease states and some normal sera as well.

In this presentation we describe the antibody nature and tissue specificity of this fluorescent

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staining pattern. It is clear that the sera under study have antibodies directed against endothelial cell cytoplasm. The antigen appears to be an interspecies antigen, similar to but not identical with a typical Forssman antigen. Whatever the chemical nature of the antigen involved, the antibody specificity directed against endothelium has some added interest in view of the fact that endothelial changes seem to be an important component of a number of vasculitic disorders as well as possibly organ transplant rejection.

MATERIALS AND METHODS

The initial serum samples examined in this series were among those submitted to this laboratory for tests for antinuclear factor (ANF), smooth muscle or striated muscle antibody activities. Consequently, this material came from a population with a high incidence of connective tissue disease. The finding of a peritubular staining pattern (PTS) in some of these sera prompted the further investigation of sera from patients with other diseases and from normal individuals. These latter samples were provided at random by the Barnes Hospital blood bank.

Fluorescent antibody technique

The indirect immunofluorescent technique was used (Coons & Kaplan, 1950). Mouse kidney (or, on occasion mouse, rat or even human liver or kidney) was used as a tissue substrate. Sections 4 μ thick were cut from frozen tissue blocks and were fixed for 10 min in acetone. Endocardial scrapings were treated in a similar fashion. Test sera were screened initially at a 1:5 dilution. The conjugated anti-human-immunoglobulin sera were prepared by the method described by Nairn (1962) and in some cases commercially prepared (Hyland Laboratories, Los Angeles, California) antiserum conjugates were used. The specificity of the reagents used was confirmed by gel diffusion and by specific absorption or blocking procedures. Slides were examined with a Leitz Ortholux microscope equipped with an Osram HBO-200 W light source, UG1 or BG12 exciting and K430 barrier filters.

Isolation of serum components

The proteins producing the endothelial staining pattern were partially purified using ammonium sulphate fractionation, followed by DEAE chromatography or by preparative zone electrophoresis. Fractions were analysed by cellulose acetate electrophoresis and by immunoelectrophoresis.

Demonstration of C′3 fixation

Fresh human serum known to be negative for peritubular staining was mixed with a positive serum that had been heat-inactivated at 56°C for 30 min. The mixture was then used for the immunofluorescent staining procedure with the exception that the fluorescein conjugate used was anti-human C′3.

RESULTS

Fig. 1 illustrates the typical endothelial staining pattern using a mouse kidney substrate. Specific fluorescence is seen around the outer border of renal tubules. This pattern was

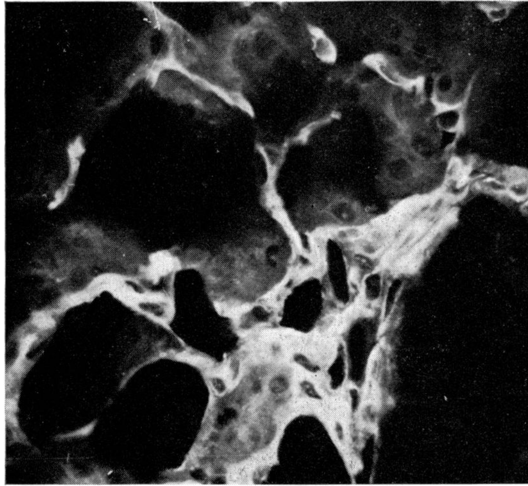

FIG. 1. The peritubular staining pattern. Mouse kidney sections treated first with human serum followed by fluorescein-tagged anti-human γ-globulin.

initially regarded as peritubular basement membrane staining. Since many of the sera examined came from patients with connective tissue diseases, several instances of sera showing both nuclear and endothelial staining were noted. However, no correlation was found between positive ANF tests and endothelial staining.

The frequency of endothelial staining in patients without evidence of connective tissue disease, was determined in 129 sera from hospitalized patients. An incidence of 17·8% was found. In a group of sixty patients with chronic pulmonary tuberculosis the incidence was 26·6% (Lindqvist, Coleman & Osterland, 1970). There was no predeliction for a particular

TABLE 1. Serum from a group O individual absorbed with erythrocytes from various species, and with blood group substances A and B

	Serum after absorption				
	Agglutination tests with				
Serum absorbed with	Human A	Human B	Sheep RBC	Rabbit RBC	Endothelial Staining
No absorption	+	+	+	+	+
Human O, Rh+	+	+	+	+	+
Human A, Rh+	−	+	+	+	+
Human B, Rh+	+	−	+	+	+
Sheep RBC	+	+	−	+	−
Rabbit RBC	+	+	+	−	−
Group A substance*	−	+	+	+	+
Group B substance*	+	−	+	+	−

* Chas. Pfizer & Co., Inc.

sex or age group. An incidence of endothelial staining was found in fourteen of one hundred normal blood-donors.

Relationship to blood groups and heterophile antigens

All human ABO blood group types were represented in the material and there was no relationship between isoagglutinin titres and the presence of endothelial staining. For example of fifty-nine patients with positive reactions who were typed, thirty-four were blood group O, nineteen group A, four group B, and two group AB. Absorption studies using human red cells confirmed the lack of correlation between endothelial antibody and ABO blood group isoagglutinins. Washed human A, B and O, Rh positive and negative erythrocytes were mixed with the serum which had been inactivated for 30 min at 56°C (0·5 ml packed erythrocytes per 0·5 ml serum) and incubated for 30 min at 37°C and overnight at 4°C. This absorption procedure was repeated once, and the serum examined for completeness of absorption by the agglutination test. These experiments did not result in any reduction in the intensity of the peritubular staining pattern (Table 1). Furthermore, no inhibition of staining was observed when N-acetyl-glucosamine, N-acetyl-galactosamine, d-galactose, fucose or mannose was added to serum in amounts up to 100 mg per ml. A mixture of these sugars at a concentration 20 mg of each per ml serum also failed to inhibit the reaction.

The staining activity would be absorbed by red cells and tissue extracts of some heterologous species. The staining was completely abolished after absorption with sheep or rabbit erythrocytes and with blood group B substance derived from gastric mucosa of the horse. Blood group A substance (Chas. Pfizer & Co., Inc.) from hog gastric mucosa had no effect (Table 1). Since the human B cells did not absorb the activity while the equine-derived B substance did, it seems apparent that the absorbing material of the latter is some component of the tissue extract other than B antigen.

Substrate specificity

The particular staining property described here was seen initially using mouse kidney sections as substrate. A lack of organ specificity was obvious since several other mouse tissues could be used as tissue substrate and yield a similar linear type staining pattern. Though the specific fluorescence was observed largely to coincide with vascular structure of the kidney, the appearance in some areas suggested that the basement membrane might be involved. However, when endothelial cells from guinea-pigs were obtained by scraping the endocardium and great vessels and subsequently stained, the cytoplasm of these cells showed bright immunofluorescent staining (Fig. 2). Close observation of staining on other tissues also suggests that the fluorescence was localized to capillary walls and vascular endothelium (Fig. 3).

Mouse, rat, human, guinea-pig and rabbit kidney sections could be stained in a similar fashion but in general, human kidney gave less conspicuous staining and so was not used for routine screening tests. Fixation for 15 min in 95% methanol prevented the staining while 95% ethanol fixation had little or no effect. In contradistinction, human blood group antigens in tissues are said to be sensitive to fixation in 95% ethanol (Szulman, 1960).

Antibody nature of the reaction

The use of fluorescent antisera specific for the major immunoglobulin classes established

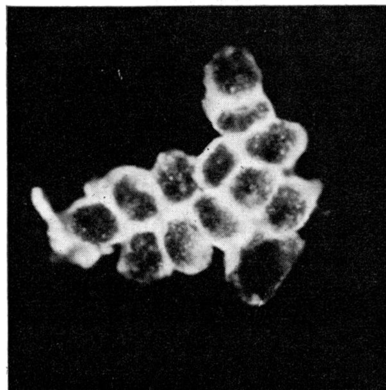

FIG. 2. Specific staining of endocardial cells. Smear of scrapings from guinea-pig endocardium was treated with human serum and fluorescein-tagged anti-human γ-globulin.

that both γG and γA at least were involved in the reaction. Controls using conjugated antibodies to albumin and fibrinogen gave negative results. A strongly positive serum was fractionated by DEAE-cellulose column chromatography, Sephadex G-200 gel filtration and agarose electrophoresis. Most of the activity was found in the γG globulin fraction. In some cases it appeared that the electrophoretically fast γG had greater activity than the slow.

Fixation of C′3

To further ensure the antigen–antibody nature of the reaction, evidence of complement fixation was sought. Since the fixation of C′3 is one of the constant secondary phenomena of antigen–antibody interaction, its participation in the process would be a strong indication

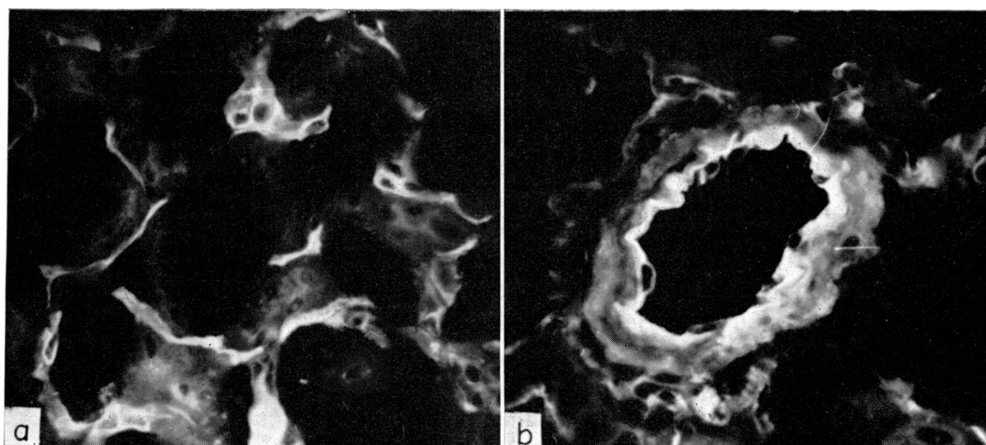

FIG. 3. Specific fluorescence localized to capillaries (a) and vascular endothelium (b). The mouse kidney sections were treated with human serum and fluorescein-tagged antihuman γ-globulin.

that the immunoglobulins responsible for the staining patterns were functioning as antibodies. Evidence of fixation of C′3 was obtained by immunofluorescence (Fig. 4). The controls, normal serum and inactivated anti-endothelial human serum, did not show any staining.

DISCUSSION

Antibody activity, revealed by the fluorescent antibody technique has been detected against vascular endothelium. Since no serum and tissue from the same patient were obtained it is not certain whether this represents auto- or iso-antibody activity. Neither the precise stimulus for production of the antibody, nor its pathogenetic significance is clear. The results show that the antibody activity is present in human serum in a variety of disease states and also in some normal individuals. No evidence was obtained to indicate that basement membrane antigens were involved in the reaction, but with this microscopical technique basement membrane and endothelial staining can look very similar.

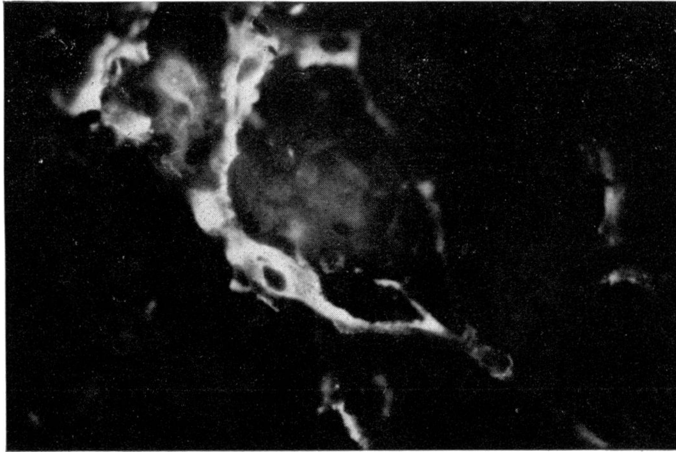

FIG. 4. Mouse kidney sections showing staining of capillary cells using fluorescein-tagged anti-human C′3 serum. Inactivated positive human serum was mixed with fresh human serum and applied to the kidney section, followed by anti C′3 conjugate.

A spontaneously occurring antibody reacting with the cytoplasm of glomerular cells has recently been described (Whittingham, Mackay & Irwin, 1966). The antibody was present in a large proprotion of sera from cases of chronic active hepatitis, and was concomitant with antibodies to smooth muscle. Apparently glomerular cells were the only cells reactive in the immunofluorescence test. The authors suggest that the antiglomerular antibody may account for the mild membranous glomerular lesions occasionally demonstrated in chronic heptatitis. Our experience with smooth muscle staining in chronic active hepatitis is at variance with the observations cited above. Of a large number of serum samples from patients with lupoid hepatitis and smooth muscle antibodies, few showed staining of glomeruli or peritubular endothelial cells.

Immunofluorescent staining of vascular endothelium and erythrocytes has been described

using calf thyroid as substrate with sera from patients with infectious mononucleosis and a positive Paul-Bunnell test (Johnson & Holborow, 1963). Wide species reactivity was not found with these sera unlike the reaction described in the present communication. The development in mononucleosis of antibodies against a herpes-like virus has been demonstrated by immunofluorescence tests using cultured Burkitt lymphoma cells (Henle, Henle & Diehl, 1968; Niederman *et al.*, 1968). These antibodies were shown to be distinctive from heterophile antibodies. It might be of interest to examine Burkitt lymphoma cells by immunofluorescence using sera giving the endothelial staining pattern.

The relationship between the antibody giving endothelial staining and isoagglutinins, Forssman antibodies and other heterophile antibodies has not been defined. While the activity seems to be a type of heterospecific antibody, its specificity is atypical and also unlike that of human blood group antibodies. Blood group antigens A and B are widely distributed in nature and in various human tissues including the intimal lining of the capillary bed (Szulman, 1960). In embryological development, vascular intimal cells take origin from the same precursors that give rise to the haematopoietic series and so a rather similar immunochemical makeup might be expected among these cell types. It is notable that high-titred anti-A and anti-B sera did not react with mouse kidney sections. Apparently, such tissues lack the human A and B antigens. Fixation of tissue sections in 95% ethanol, which has been shown to abolish staining of the vascular intima of man by potent anti-A and anti-B sera (Szulman, 1960), had only a small effect on the endothelial staining pattern observed in our material. However, fixation in 95% methanol abolished the staining. Sera giving endothelial staining came from individuals of all ABO blood groups, and absorption with A, B and O cells had no effect on the staining property.

It is also apparent that the antibodies are not of the Forssman type, since rat and rabbit kidney lacking the Forssman antigen (Boyd, 1966) can serve as substrates, and some human sera from group A and AB individuals which possess the Forssman antigen contained antibodies to endothelial cells. The antigen is present on sheep and rabbit red cells and in group B substance isolated from horses, since absorption with these completely abolished the endothelial staining.

The antigen responsible for this staining pattern appears to be present in a variety of animal species. Whether it is present in every human individual or absent in individuals whose serum contains the corresponding antibodies is not certain. While human kidney can be used as the tissue for demonstrating this activity the pattern visualized is not as striking as with mouse or rat kidney. It is not known if the production of the antibodies is stimulated by endogenous factors and host antigen thus representing an autoimmune phenomenon, or if their presence is due to antigenic stimulation of foreign origin, for example infectious agents. The cross-reactivity of the antibodies with tissues and red cells of different species seems to justify their designation as heterophile antibodies (Jenkin, 1963).

Several investigators have described the formation of isoantibodies and autoantibodies by patients who had received organ transplants (Wasaki, Talmage & Starzl, 1967; Porter *et al.*, 1964; Klassen & Milgrom, 1969). Immunologic observations on renal homografts have confirmed that both γ-globulin and C′3 are localized to vascular endothelium during graft rejection (Horowitz *et al.*, 1965). The work of Rapaport, Kano & Milgrom (1968) on the formation of heterophile antibody in patients receiving transplants seems especially pertinent to the endothelial antibodies described here.

The presence of these antibodies in normal persons as well as in a variety of disease states

suggests that they bear no direct relationship to clinical disease. In the normal, intact endothelial cell, the antigen may not be accessible to the antibodies; additional factors may be required for these antibodies to assume a pathogenetic role. At present it cannot be stated whether surface or only intracellular antigens are involved.

The demonstrations of anti-endothelial activity in sera from two transplant patients may be significant in view of the reports of antibodies and complement on endothelial cells during graft rejection. It seems plausible that antibodies to vascular endothelium constitute an early immunological attack on grafts and they may be concerned in accelerated graft rejection phenomena (Lindqvist, *et al.*, 1970; Szulman, 1960).

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