THE LOCALIZATION OF IN VIVO BOUND COMPLEMENT IN TISSUE SECTIONS

By P. J. LACHMANN, M.D., H. J. MÜLLER-EBERHARD, M.D., H. G. KUNKEL, M.D., and F. PARONETTO, M.D.

(From The Rockefeller Institute, and the Department of Pathology, Mount Sinai Hospital, New York)

Plates 8 to 11

(Received for publication, August 23, 1961)

The possibility that autoclastic immune reactions are causally involved in some human diseases has led to a search for evidence of immunological phenomena in the lesions of such diseases. The demonstration, by immunofluorescence, of bound γ-globulin (1) was the first, and till recently, almost the only technique available for this purpose. By this method, bound γ-globulin has been found in the lesions of experimental immune disorders such as experimental serum sickness (2) and experimental nephritis (1): and in the lesions of human diseases such as systemic lupus erythematosus (L.E.) glomerulonephritis, amyloidosis, and some of the other connective tissue disorders (3–7). Attempts have been made to show that such bound γ-globulin is likely to be antibody by showing its presence independent of other serum proteins such as albumin and fibrinogen (8), and by eluting the γ-globulin at acid pH (9).

There remains, however, a wide gap between the demonstration of bound γ-globulin in a lesion and the implication of immune reactions as pathogenic factors. It has been widely felt that one step towards closing this gap would be the demonstration of complement in the lesions, since it has been found (10) that the fixation of complement on tissue cells produces cytotoxic changes. To apply these findings to the lesions of disease, it is of course necessary to detect the patient's own complement that has been bound *in vivo*.

The possibility of demonstrating the patient's own *in vivo* bound complement has recently become feasible following the purification from human serum of β_{1C}-globulin, a moiety of the third component of complement (11). Since β_{1C} is strongly antigenic and is, in part, fixed on immune precipitates; and also since its fixation is one of the last steps in complement binding, it provides a highly suitable antigen for studying complement fixation by immunofluorescence. An immunologically pure anti-β_{1C} serum has been prepared in rabbits, a fluorescein conjugate of which serves as a specific reagent for the detection of the bound C′ component.

A number of studies have appeared on the localization of complement, added *in vitro*, to tissue sections. These stem from the work of Goldwasser and Shepard (12)

who were interested in using a single fluorescein conjugate in conjunction with antibodies from different species. The method was further developed and applied to the examination of experimental lesions by Klein and Burkholder (13); and subsequently Vogt and Kochem (14) have successfully applied it in human amyloid and Strauss and his colleagues (15) in myasthenia gravis. The principle of the Goldwasser method is to fix guinea pig complement onto tissue sections from other species, using standard serological controls to show that the fixed material is specifically complement. A fluorescein-conjugated "anti-guinea pig complement" serum is then used to detect the fixed material. The exact specificity of this antiserum is unimportant and equally good results have been obtained (13) with a crude anti-guinea pig globulin serum as with more defined antisera.

The demonstration of *in vivo* bound complement represents a totally different problem. Not only is there no control over the conditions under which the complement is fixed, but the complement is of the same species as the rest of the tissue. This leaves the precise antigenic specificity of the "anti-complement" serum as the only factor from which the specificity for complement of the staining can be inferred. Although attempts to make "anti-complement" sera go back for many years, such sera usually contained antibodies against multiple antigens and no firm evidence has been produced that they reacted exclusively with complement components. Such reservations notwithstanding, evidence has been adduced by the use of anti-complement sera for the presence of *in vivo* bound complement in lupus nephritis by Freedman and Markowitz (16), and in experimental nephritis in the rat by Klein and Burkholder (13).

Using a fluorescein conjugate of an immunologically pure anti-β_{1C} globulin serum, the question of the occurrence of *in vivo* bound complement and its relation to *in vitro* complement fixation and to the presence of some other serum components in lesions has been investigated in a number of diseased human tissues. In the present communication the methods and the initial results are presented.

Materials and Methods

Preparation of Sections.—Tissues were obtained at postmortem, within 12 hours of death, or by biopsy. The tissues were cut into suitable size blocks and either frozen at once in a dry ice and acetone mixture, or embedded in gelatin before freezing as described by Burkholder, Littell, and Klein (17). In either case the frozen blocks were then stored at −20°C.

Sections were cut at a thickness of 4 to 6 microns in a Harris International cryostat, dehydrated, first by air-drying and then in a vacuum desiccator for 30 minutes, and then fixed in acetone for 10 minutes at 4°. They were then stained immediately or stored at −20° for up to 3 days.

Hematoxylin and eosin–stained preparations were obtained for all tissues, either from formalin-fixed tissue taken at the same time, or by fixing sections from the cryostat in formalin. Where indicated, other histological techniques were also used.

Antisera.—All antisera were prepared in rabbits. A predominantly γ-globulin fraction of the sera was prepared by a single precipitation with 15 per cent sodium sulfate. The precipitated globulins were washed once in 15 per cent sodium sulfate, redissolved in isotonic phosphate buffer, pH 8.0, and then dialyzed against phosphate buffered saline, pH 7.3 (PBS).

Conjugation with fluorescein isothiocyanate (Baltimore Biologicals) was carried out substantially as described by Marshall, Eveland, and Smith (18). The conjugates were freed from excess fluorescein either by dialysis or by filtration through sephadex (Pharmacia). The latter method gives a very good separation of the conjugate from the free dye and can be completed in a few hours. Before use the conjugates were absorbed with guinea pig liver powder.

All globulin solutions were stored at 4° with $\frac{1}{1000}$ sodium azide. Estimations of protein concentration [P] were made by the Folin method. The concentration of fluorescein [F] in the conjugates was measured approximately by their optical density of 495 mμ and comparison with a standard curve (12).

The following conjugates have been used:

Anti-β_{1C}-Globulin.—The antiserum was prepared by immunization with pure β_{1C}-globulin (prepared as previously described (11)). The conjugate was used at protein [P] = 13.6 mg/ml and fluorescein [F] = 35 μg/ml. The fluorescein:protein ratio [F]/[P] = 2.6×10^{-3}. The conjugate gave a single characteristic β_{1C}–β_{1A} line in immunoelectrophoresis (Text-fig. 1).

Anti-7S γ-Globulin.—The antiserum was prepared by immunization with γ-globulin aggregates prepared by heating human Cohn fraction II. The conjugate was used at [P] = 9.6 mg/ml and [F] = 25 μg/ml. [F]/[P] = 2.6×10^{-3}. The conjugate gave a single, characteristic γ-globulin line in immunoelectrophoresis (Text-fig. 2).

Anti-19S γ-Globulin.—The antiserum was prepared by immunization with the isolated macroglobulin from the serum of a patient with Waldenström's macroglobulinemia. The conjugate was used at [P] = 10.2 mg/ml and [F] = 22.5 μg/ml [F]/[P] = 2.2×10^{-3}. The conjugate gave a strong characteristic macroglobulin line on immunoelectrophoresis with normal serum and a faint anti-7S γ-globulin line (Text-fig. 2). To avoid lowering the antibody titer, the antiserum was not absorbed with 7S γ-globulin, and a blocking technique was used on the sections to control specificity.

In the optical system used these three conjugates gave virtually no background staining and good specific fluorescence.

Anti-fibrinogen.—The antiserum was prepared by immunization with commerically available fibrinogen (pentex Fr. I). It was absorbed with human serum. The conjugate was used at [P] = 25 mg/ml and [F] = 200 μg/ml. [F]/[P] = 8.0×10^{-3}. The conjugate gave a single faint line on immunoelectrophoresis (Text-fig. 1). This conjugate gave a moderate degree of background staining but its specific fluorescence was very bright. The low antibody activity made it necessary to use the serum at the high protein concentration and consequently high levels of fluorescence. For our optical system this serum was less satisfactory than the others.

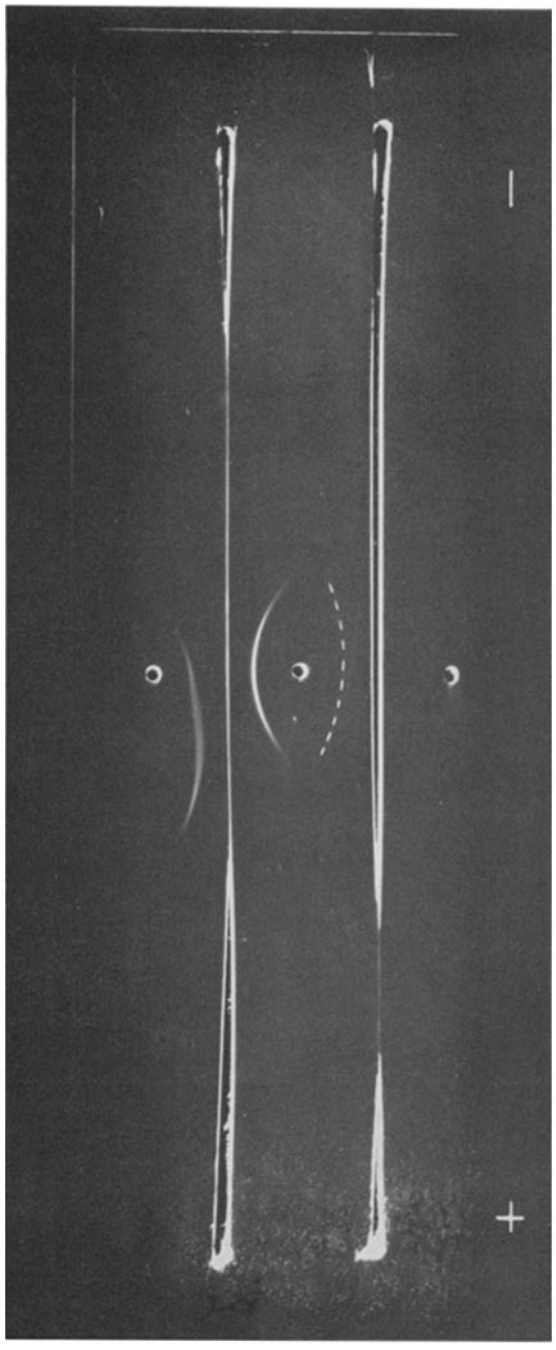
Optical System.—A Leitz labolux microscope was used with an Osram HBO 200 watt mercury lamp, a 4 mm UGI activating filter and a D 120 A dark-field immersion condenser. Microscope slides were of 1 mm thickness.

Evaluation of Staining Reactions.—Staining was assessed subjectively on the following graded scale:

Negative	Feeble	Moderate	Bright	Very bright
0	±	+	++	+++

Immunofluorescent Staining Methods:—

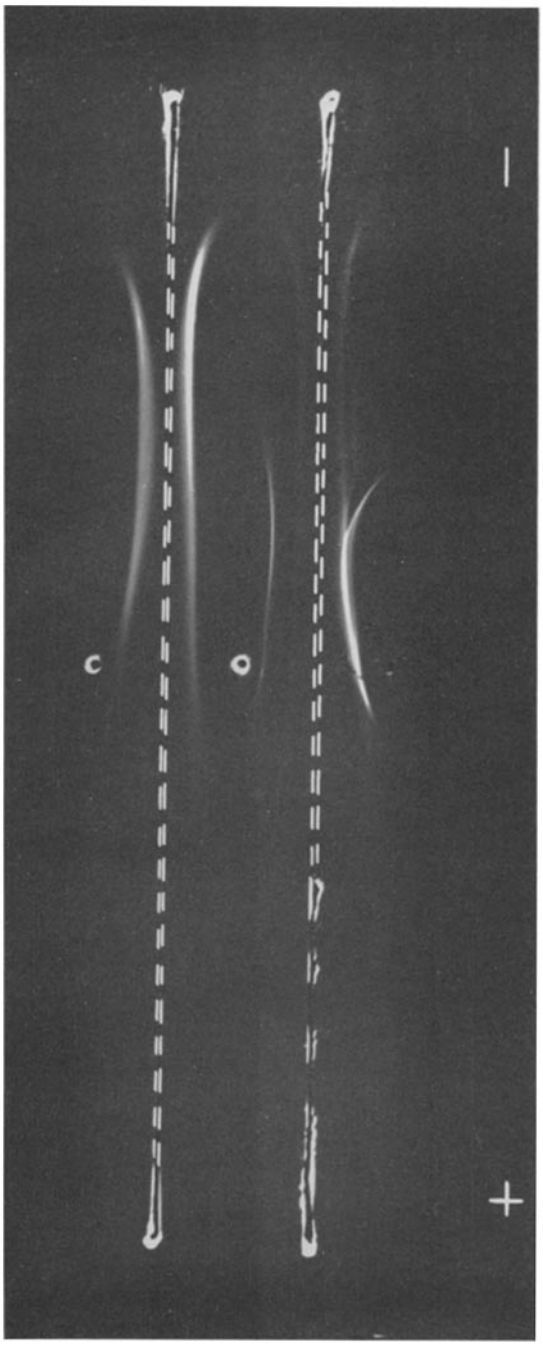
To demonstrate β_{1C}-globulin, 7S γ-globulin or fibrinogen: One section, three times washed in PBS was stained for 30 minutes at room temperature with the conjugate. A control section was incubated similarly with the corresponding unconjugated globulin solution (at a protein



TEXT-FIG. 1. Immunoelectrophoretic patterns developed with anti-β_{1C} and anti-fibrinogen conjugates.

Top well: hydrazine-treated human serum
Upper trough: anti-β_{1C} conjugate
Middle well: fresh human EDTA plasma
Lower trough: anti-fibrinogen conjugate
Bottom well: fresh human serum

The anti-β_{1C} conjugate gives the faster line characteristic of β_{1A} with the hydrazine-treated human serum and the typical β_{1C} line with the EDTA plasma (11). The anti-fibrinogen conjugate gives a faint line (dotted in on the photograph) with the EDTA plasma and no line with serum.



TEXT-FIG. 2. Immunoelectrophoretic patterns developed with anti-β_{2M} and anti-7S γ globulin conjugates.

Top well: human fraction II γ-globulin
Upper trough: anti-7S γ-globulin conjugate
Middle well: fresh human serum
Lower trough: anti-19S conjugate
Bottom well: serum B (high titer rheumatoid factor)

The anti-19S conjugate gives a characteristic macroglobulin line with the normal serum. With serum B this line is heavier. A faint anti-γ-globulin line is seen in both cases. The anti-7S γ-globulin conjugate shows a single typical γ-globulin line against human serum and against fraction II.

concentration two to three times that of the conjugate) before adding the conjugate, the section not being washed between the two incubations. In occasional cases three successive additions of unconjugated globulin were made instead of just one before adding the conjugate. A difference of two grades or more in the intensity of fluorescence of the two sections was taken to indicate "specific staining."

To demonstrate 19S γ-globulin: To eliminate the reaction with 7S γ-globulin the test section is pretreated with unconjugated anti-7S γ-globulin before adding the anti-19S conjugate The control section was pretreated in the usual way with unconjugated anti-19S γ-globulin.

Indirect Rheumatoid Factor Staining.—

The test section was treated for 30 minutes at room temperature with diluted serum B containing a uniquely high titer of rheumatoid factor or isolated rheumatoid factor. It was then washed three times in PBS and stained with anti-19S conjugate. The two sections used in the detection of 19S above acted as controls for this system. Only if bright 7S γ-globulin staining had been found was blocking of the test section with unconjugated 7S γ globulin necessary before staining.

The procedure for the model system in Table II was as follows: Substrate was treated with source of antibody for 30 minutes at room temperature, washed three times, and then treated with source of rheumatoid factor for 30 minutes at room temperature. After three further washes the slides were treated for 30 minutes at room temperature with unconjugated anti-7S γ-globulin, and were then stained with the anti-19S conjugate.

To demonstrate in vitro complement fixation: Sections were incubated for 30 minutes at 37° with a fresh normal human serum known to be free of rheumatoid factor, immunoconglutinin and antibodies to human tissue antigens demonstrable by complement fixation or immunofluorescence. The sections were then washed three times in PBS and stained with anti-β_{1C} globulin conjugate. In the experiments using reagents for the detection of complement components these were prepared by standard methods (19, 20).

Immunoelectrophoresis.—This was carried out according to Scheidegger (33).

RESULTS

To Show in a Model System That the Fixation of β_{1C} Detected by Immunofluorescence is Comparable to Its Behavior in a Hemolytic System.—In a hemolytic system (10) β_{1C} represents the hydrazine sensitive moiety of C'_3 (the third component of complement) and reacts with the complex EAC'_{0142} (antigen-antibody-11S component-first, fourth, and second complement components). It does not react with EA (antigen-antibody complexes) or any of EAC' complexes formed before EAC'_{0142}. The fixation of β_{1C} on EAC'_{0142} occurs best at 37° and is inhibited at 4°. It does not require Ca or Mg ions.

For testing β_{1C} fixation by immunofluorescence the following system was employed. Sections of normal human liver were treated with a globulin fraction of a lupus serum containing high titer complement-fixing antibodies to cell nuclei. After washing, the sections were treated with various complement reagents, washed, and then stained with anti-β_{1C} conjugate. Table I shows the results obtained.

It can be seen that the results correspond exactly to the behavior of β_{1C} in the hemolytic system as described above. In the immunofluorescent system the

TABLE I

A Model System to Show the Specificity of the Immunofluorescent Determination of β_{1C}-Globulin

Normal liver sections, treated for 30 min. at 25° with lupus globulin (P 10 mg/ml) washed and treated with:—		Sections then washed and stained for 30 min. at 25° with conjugates of:—	Nuclear fluorescence
Phosphate buffered saline	30 min., 37°	Anti-7S γ-globulin	++
" " "	" " , "	Anti-β_{1C}-globulin	0
Fresh human serum	30 min., 37°	Anti-β_{1C}-globulin	++
" " "	" " , 25°	" " "	+
" " "	" " , 4°	" " "	0
Fresh human serum + 0.01 M EDTA	30 min., 37°	Anti-β_{1C}-globulin	0
Human serum, heated 56° for 30 min.	" " , "	" " "	0
R_{11S}	30 min., 37°	Anti-β_{1C}-globulin	±
R_1	" " , "	" " "	±
R_2	" " , "	" " "	0
$R_1 + R_2$	" " , "	" " "	++
R_4	" " , "	" " "	0
$R_4 + \beta_{1C}$-globulin	" " , "	" " "	0
R_3 (standard reagent)	" " , "	" " "	++
R_3 (reabsorbed with excess zymosan)	" " , "	" " "	0
Guinea pig R_3	" " , "	" " "	0
" " " + β_{1C}-globulin	" " , "	" " "	++
β_{1C}-globulin	" " , "	" " "	0
Normal liver sections (no antibody added washed and treated with:—			
Fresh human serum	30 min., 37°	Anti-7S γ-globulin	0
" " "	" " , "	Anti-β_{1C}-globulin	0
Smear of heat-aggregated human fraction II γ-globulin washed and treated with:—			
Fresh human serum	30 min., 37°	Anti-β_{1C}-globulin	+++
Heated " "	" " , "	" " "	0

reagents are used undiluted, and those which are more difficult to prepare satisfactorily (R_{11S} and R_1) retained feeble staining although they were satisfactory for use in hemolytic assays. As β_{1C}-globulin is itself hydrazine-sensitive it is necessary to add it to an R_4 before concluding that C'_4 is essential for β_{1C} fixation. A hemolytically inert C'_3 retained bright staining which was, however, removed by a further absorption with zymosan. Immunoelectrophoresis showed that the standard R_3 contained a little β_{1C}-globulin, while the reabsorbed reagent contained only the inactivated form β_{1A}-globulin. An R_3

reagent made from guinea pig serum gave, of course, no staining, but on addition of β_{1C}-globulin bright staining was obtained. β_{1C}-globulin on its own gives rise to no staining.

In the absence of antibody, the complement reagents did not lead to β_{1C} fixation on the liver sections. Smears of heat-aggregated γ-globulin, on the other hand, would act alone as substrate for complement fixation. β_{1C}, once fixed, was not removed by treatment of the section with EDTA, nor by elution with pH 3 buffer for 10 minutes at room temperature.

TABLE II

Model system to show the specificity of 'Indirect Rheumatoid Factor' staining

Substrate	Source of antibody	Source of rheumatoid factor (R.F.)	Staining
Normal liver section	Phosphate buffered saline	None	0 nuclei
" " "	" "	Serum B, (high titer R.F.)	0 "
" " "	" "	Isolated R.F.	0 "
" " "	" "	Normal serum	0 "
Normal liver section	Lupus globulin (patient) = 10 mg/ml	None	± nuclei
" " "	" "	Serum B	++ "
" " "	" "	Isolated R.F.	++ "
" " "	" "	Normal serum	± "
Smear of heat-aggregated human fraction II γ-globulin	None	None	± smear
" "	"	Serum B	++ "
" "	"	Isolated R.F.	++ "
" "	"	Normal serum	± "

A similar model system was used to show the specificity of the indirect rheumatoid factor staining for antigen-antibody complexes and aggregates. Table II shows the results.

The Demonstration of in Vivo Bound β_{1C}-Globulin in Tissue Sections.—The tissues examined for the presence of β_{1C}-globulin and 7S γ-globulin are presented in Table III to VI. For convenience the material is presented according to diagnosis. Only specific staining is recorded. Table III shows the results obtained with material from cases of systemic lupus erythematosus. Staining with anti-β_{1C} conjugate was found predominantly in the glomeruli affected by lupus nephritis in which the tufts still persisted. Hyalinized and fibrosed glomeruli showed little staining. The walls of small vessels in the kidney and

TABLE III

Specific β_{1C}-Globulin and 7S γ-Globulin Staining in Tissues from Patients with Systemic Lupus Erythematosus

Patient	Tissue	Histological picture	Features stained by: Anti-β_{1C}-globulin		Anti-7S γ-globulin
A	Kidney	Lupus nephritis	+++	Glomeruli	+++
			+++	Occasional vessel	+++
			0	Casts	++
			0	Plasma cells	++
	Lymph node	Increased plasma cells	0	Plasma cells	++
	Liver	No lesions	0		0
	Spleen		+	Vessels	+
			0	Occasional plasma cell	++
B	Liver	No lesions	0	Connective tissue in portal tract	±
	Lymph node	Increased plasma cells	0	Plasma cells	++
C	Spleen	Onion skin lesions (rare)	++	Plasma cells	++ (many)
			+	Intima of some vessels	+
	Heart	No lesions	+	Amorphous bodies	+
D	Renal biopsy	Slight interstitial fibrosis; glomeruli not obviously abnormal	++	Patches of glomerular basement membrane	+
E	Renal biopsy	Severe glomerular disease	±	Glomerular basement membrane	0
			±	Intima of vessel	+
			0	Casts	++
F	Spleen	Chronic vasculitis	+	Intima of vessels	++
	Liver	Severe fatty change	0	Intracellular granules	±
	Heart	No lesions	0		0
	Kidney	Lupus nephritis	++	Patches of glomerular basement membrane	+
			+	Occasional vessel	++
			0	Casts	+++
	Thyroid	No lesions	0		0

spleen were also stained. In these sites β_{1C} staining ran generally parallel to γ-globulin staining. In anatomically "normal" parts of the tissue β_{1C} was not found. Most renal casts and the great majority of plasma cells did not stain with antiβ_{-1C} globulin. However, in a lupus spleen a small proportion of the

plasma cells did stain specifically with β_{1C} antiserum. The term "plasma cell" in this connection describes a cell of regular oval shape, with a single eccentric nucleus and a non-granular cytoplasm.

TABLE IV

β_{1C} and 7S staining in "nephritis"

Patient	Clinical diagnosis	Histological picture in kidney	Features stained by		
			Anti-β_{1C}-globulin		Anti 7S γ-globulin
G	Subacute glomerulonephritis	Typical histology	++	Glomerular tufts	++ (basement membrane)
			++	Afferent arterioles	++
			0	Plasma cells	++
			0	Casts	+++
H	Subacute glomerulonephritis	Membranous glomerular lesions	++	Glomeruli	+++
			0	Plasma cells	++
I	Relapsing glomerulonephritis (in remission)	Some swelling of glomeruli	+ (rare streaks)	Glomerular tufts	+ (throughout)
J	Hypertension	Arteriosclerosis chronic pyelonephritis	+	Glomerular tuft	+
			+	Thickened intima of vessels	+
K	Acute nephritis	Some swelling of glomeruli. Increased cellularity of tufts	+ (throughout)	Glomeruli	++ (throughout)
			+	Occasional small vessel	+
L	Generalized arteriosclerosis	Chronic pyelonephritis	+	Glomeruli	+
			+	Intima of some vessels	+
			0	Casts	+++
M	Polyarteritis nodosa	Focal glomerular necrosis (No typical polyarteritis lesions in block examined)	+	Occasional vessel	+
			0	Plasma cells	++
			+	Streaking in glomerular tufts	±
N	Malignant nephrosclerosis	Necrotizing arteriolitis focal glomerular necrosis	+ (patchy)	Glandular tufts	++ (throughout)
			++	Occasional vessels	++
O	Birth injury	Normal	0		0

Table IV shows the results on kidney sections from cases of "nephritis"—using this term widely. Bright β_{1C}-globulin staining was found in the glomeruli of the two cases of subacute glomerulonephritis and in one of these cases there was some staining of the intima of the afferent arterioles as well. Staining of glomeruli in the three cases of acute glomerulonephritis was less strong but the material here was in no case obtained at the height of the disease. Some staining was found in the glomeruli of the single cases of chronic pyelonephritis, polyarteritis nodosa, and malignant nephrosclerosis. In all these cases, again, the

staining occurred only when there were anatomical lesions and it was more or less parallel with the staining of γ-globulin.

Table V shows the results on 3 cases of amyloid that were studied. One was of primary type and involved the heart predominantly, another was also regarded as primary although spleen and liver were involved. The third case was of secondary type with the main involvement in kidney and liver. All three cases showed bright staining of the amyloid for β_{1C}-globulin. In other respects, however, there were differences among the amyloid deposits. In one case of the three the unstained deposits showed a bluish autofluorescence. In this case and one of the other two there was staining with the anti-7S γ-globulin serum. In the

TABLE V

β_{1C}-Globulin and 7S γ-Globulin Staining in Amyloid

Patient	Tissue	Histological picture	Features stained by: Anti-β_{1C}-globulin		Anti-7S γ-globulin
P	Spleen	Well marked amyloid deposits around vessels	++	Amyloid deposits	++*
			0	Plasma cells	++
	Liver	No amyloid deposits	0		0
Q	Heart	Amyloid deposits between muscle	++	Amyloid deposits	++
R	Renal biopsy	Amyloid in glomerulus in walls of vessel and around tubules	++‡	All amyloid deposits	0
			0	Casts	++

* 7S γ-globulin is more extensive than β_{1C}.

‡ Requires three applications of inhibiting serum to show specificity.

third case there was no γ-globulin staining and it needed three applications of unconjugated antiserum to inhibit the anti-β_{1C} staining. This was interpreted as indicating a high concentration of β_{1C} at the binding site. This last case was studied further and is discussed again below.

Table VI shows the results in a miscellany of other pathological tissues studied. Most of these show no β_{1C} staining. However, positive staining was found in the intima of the smaller arteries of several tissues of a patient with Waldenström's macroglobulinemia. These vessels were not obviously abnormal on conventional histology and did not stain with anti-7S γ-globulin. One of these tissues was studied in greater detail and will be discussed again.

The spleen of a child with idiopathic thrombocytopenic purpura and hemolytic anemia showed staining of the intima of small vessels with both anti-β_{1C} and anti-7Sγ-globulin. The reticulum around the vessels stained with anti-β_{1C}

TABLE VI

β_{1C}-and 7S γ-Globulin Staining in Miscellaneous Diseases

Patient	Clinical diagnosis	Tissue	Histological picture	Features stained by: Anti-β_{1C}-globulin		Anti-7S γ-globulin	Comments
S	Acute rheumatic fever	Lung	Edema, some hyaline membranes in alveoli	+ +	"Streaks" in septa extracellular droplets	0 +	Droplets in septa and alveoli PAS-positive. They do not stain with anti-fibrinogen
		Heart	Acute and healing Aschoff bodies	++ +	Droplets as in lung Sub-intimal layer of vessels in Aschoff bodies	++ +	
T	? Allergic angiitis	Lymph node	"Subacute lymphadenitis" with eosinophilia	0		0	See Table VII
U	Chronic thyroiditis	Thyroid	Typical histology with marked fibrosis	0		0	
V	Arteriosclerosis	Aorta	Early atheroma	0		0	Atheroma stains with anti-fibrinogen
W	"Placental insufficiency"	Placenta	"Fibrinoid" around villi	0		0	"Fibrinoid" stains strongly with antifibrinogen
X	Sarcoidosis	Lung	Typical	0		0	
Y	Sarcoidosis	Lymph node	Typical	0		0	
Z	Waldenström's macroglobulinemia	Kidney	No obvious abnormality	++	Intima of vessels	0	See also Table VII
		Spleen	Increased plasma cells	++	Intima of vessels	0	Many plasma cells stain with anti-19S
				0	Plasma cells	++ (occasional)	
AA	Idiopathic thrombocytopenic purpura and hemolytic anemia	Spleen		++ (rare)	Plasma cells	++ (moderately frequent)	Reticulum stains also with anti-fibrinogen
				++	Intima of vessels	+	
				++	Reticulum around vessels	0	
AB	Sarcomatosis	Peritoneal metastasis	Angiosarcoma	0		0	Bright staining with anti-fibrinogen
AC	Bronchopneumonia	Lung	Acute confluent bronchopneumonia with polymorph exudate	0		0	Bright staining with anti-fibrinogen
AD	Hyaline membrane disease	Lung	Typical	0		0	Bright staining with anti-fibrinogen

and with anti-fibrinogen but not with anti-7Sγ-globulin. In this spleen a few "plasma cells" were again seen to stain with anti-β_{1C}.

The heart of a patient dying of acute rheumatic fever showed staining of the intima of vessels in the Aschoff bodies with anti-β_{1C}. Many small "droplets" stained with both anti-β_{1C} and anti-7S γ-globulin. These droplets, which were extracellular, were found to stain with the periodic acid–Schiff reagents after diastase treatment and were palely eosinophilic and difficult to see on the

TABLE VII

Further Immunofluorescent Studies on Selected Tissues

Patient	Diagnosis	Tissue	Histological feature under study	Anti-β_{1C}-globulin	Anti-β_{1C} (after incubation with C′)	Anti-7S γ-globulin	Anti-19S	Indirect rheumatoid factor staining	Anti-fibrinogen
A	SLE	Kidney	Glomerular tufts	+++	+++	+++	+	+++	+ (occasionally)
			Plasma cells	0	0	++	0	0	0
			Casts	0	+	+++	+++	+++	0
Z	Waldenström macroglobulinemia	Kidney	Intima of arterioles	++	++	0	0	±	++
R	Amyloidosis	Kidney	Amyloid	++	++	0	±	+	++
T	Allergic angiitis	Lymph node	Reticulum	0	+++	0*	0	+++	+++
B	SLE	Liver	Epithelium of bile ducts	0	++	0	0	++ (also stains bile thrombi)	++ (whole sinusoid network)
F	SLE	Liver	Granules in liver cells around fat droplets	0	++	±	0	+	0

* After incubation with C′, anti-7S γ-globulin staining was still negative.

hematoxylin and eosin section. Similar droplets were seen in the septa and alveoli of the lungs of the same patient.

Sections of normal liver, kidney, heart muscle, and skeletal muscle showed no staining with anti-β_{1C} and anti-7S γ-globulin. Sections of normal spleen showed plasma cells staining with anti-7S γ-globulin, but no cells staining with anti-β_{1C}.

More Detailed Study of Selected Tissues.—Many of the sections were studied by further staining techniques. Table VII summarizes some selected results that bear upon the significance of the β_{1C} staining. In a typical case of lupus nephritis (A) the glomeruli stained with anti-β_{1C}- and anti-7S γ-globulin and to a lesser extent with anti-19S γ-globulin and anti-fibrinogen. Incubation with

fresh human serum did not alter the β_{1C} staining. The indirect rheumatoid factor staining was strongly positive.

Lack of correlation between β_{1C}- and γ-globulin staining was found in renal casts and plasma cells in which staining was obtained only with anti-γ-globulin. The plasma cells did not stain with β_{1C} after incubation with human complement nor by the indirect rheumatoid factor method. The renal casts on the other hand stained with β_{1C}', though by no means always brightly, after incubation with human C′. They, therefore, show some *in vitro* complement fixation without having complement bound on them *in vivo*. They stained brightly with the anti-19S serum, so that the rheumatoid factor staining by this technique could not be applied.

Lack of correlation between β_{1C}- and 7S γ-globulin staining was also found in the intimal vessel staining found in most of the organs studied of a case of Waldenström's macroglobulinemia and in the amyloid deposits of one of the cases of amyloid. In both of these instances, there was a correlation of staining with anti-fibrinogen. However, in many other instances, there was bright anti-fibrin staining with no β_{1C} staining. In both the instances quoted there was only feeble staining by the indirect rheumatoid factor method.

Three cases are shown where β_{1C} staining became positive only after incubation with human C′, *i.e.* where there was *in vitro* but not *in vivo* complement fixation. The most dramatic example was with a lymph node biopsy from a patient (T) with a tentative diagnosis of allergic angiitis. This lymph node showed no staining with either β_{1C}- or 7S γ-globulin. However, its whole "reticulum framework" stained very brightly with anti-β_{1C}, but not at all with anti-7S γ-globulin, after incubation with C′. A similar pattern of fluorescence was found when this node was stained by the indirect rheumatoid factor method. With anti-fibrinogen more extensive staining of the structural background of the gland was seen. A similar spectrum of staining was seen with the splenic reticulum of a case of idopathic thrombocytopenic purpura and hemolytic anemia in a child (See Table VI).

The epithelium of the bile ducts in a case of systemic L.E. and some granules in the hepatic cells of another case also showed solely *in vitro* complement staining. The former stained well by the indirect rheumatoid factor method; the latter only feebly. The former failed to stain with the anti-γ-globulin serum; the latter stained feebly.

DISCUSSION

From the evidence of the *in vitro* complement fixation studies it can be inferred that β_{1C}–globulin is fixed on tissue sections under conditions that accurately parallel those of complement fixation and that in sections, just as in a hemolytic system, it is the SAC'_{0142} complex with which it combines. On

the basis of these findings, it seems highly likely that the demonstration of β_{1C}-globulin in an untreated tissue section is indicative of *in vivo* complement fixation at that site.

From the initial survey of pathological material that has been reported, there seems to be no doubt that *in vivo* complement fixation does occur at the site of certain lesions and not in normal tissues. Furthermore, analogy with the work of Ross and Lepow (10) on the fixation of complement on antibody-sensitized tissue culture cells would suggest that such complement fixation represents a true biological complement fixation with cytotoxic sequelae.

However, the exact significance that can be attached to finding complement bound in a tissue requires continued cautious evaluation. Previous studies have tended to assume that even *in vitro* complement binding could be used as evidence for the presence of antigen-antibody complexes at the binding site. In view of the powerful binding of complement to γ-globulin aggregates (21–23), whether these be made by heating (24), treatment with mercaptoethanol-urea (25), or with bis-diazobenzidine (23), this assumption is clearly unwarranted. Smears of heat-aggregated γ-globulin have served as a substrate to show complement fixation by staining with anti-β_{1C} conjugate and very bright fluorescence was obtained. In fact, the presence of bound complement at a tissue site can be attributed to any of three situations which are known to fix complement:

(*a*) Antibody bound to antigens normally present at the site. This is the situation studied by Ross and Lepow (10).

(*b*) Antigen-antibody complexes unrelated to the tissue site and either bound there by immune adherence (26) or trapped mechanically. This is apparently the situation in serum sickness.

(*c*) γ-globulin aggregates bound at the site by one mechanism or another. Further, it appears possible that complement may be bound at the sites of immune reactions other than those involving circulating antibodies.

In experimental conditions, immune complexes can be distinguished from γ-globulin aggregates by identifying the antigen at the site of the lesion (2); but in human disease where the antigen is either normally present at the lesion site or is unknown, this approach is difficult.

In view of the finding of β_{1C} staining at sites showing no γ-globulin staining, the question arises whether in these cases complement is fixed on a substrate other than γ-globulin, or whether γ-globulin is present and is not stained either because there is too little or because it has lost its antigenicity. Complement is known to be bound on inert particles such as quartz and kaolin, and on non-immune precipitates such as those made with DNA and histone. Since complement preparations free of γ-globulin are not available it is not certain whether this binding is always preceded by γ-globulin binding. That

this is the case, however, is suggested by the observation that such systems, after treatment with a heated serum, are agglutinated by an anti–γ-globulin serum (27).

A further reason for believing that γ-globulin was present at sites where β_{1C} was fixed is based on the reaction with rheumatoid factor. The specificity of rheumatoid factor for aggregated γ-globulin and antigen-antibody complexes is well established (25, 28) and a reaction with rheumatoid factor can be interpreted as showing the presence of 7S γ-globulin, either aggregated or in an immune complex, at the reaction site. An immunofluorescent method for this purpose was used on much of the material in this study and showed that in all cases where there was β_{1C} bound *in vivo*, or where it could be bound *in vitro*, there was at least some reaction with rheumatoid factor. The method shows promise as an alternative to *in vitro* complement staining for showing antigen-antibody complexes and aggregated γ-globulin.

In vivo it may well be less important from the standpoint of pathogenicity whether complement is fixed on one substrate or another. Recent evidence clearly indicates that γ-globulin aggregates are biologically active in producing skin reactions (21, 29). An important factor appears to be whether the site of complement fixation is near enough a cell surface for pharmacologically active agents to be released. Consequently, β_{1C} fixation *in vitro* in the absence of *in vivo* binding could be without cytotoxic importance as would β_{1C}-binding away from cells.

From the material studied the following tentative conclusions could be reached. The glomerular and vascular lesions of systemic L.E. and of "nephritis" showed *in vivo* complement binding parallel in distribution with γ-globulin binding, although the relative brightness of the staining with the two reagents varied markedly from case to case. Indirect rheumatoid factor staining also followed the same pattern of distribution. In these diseases immune complexes may reasonably be implicated and the complement binding may well be producing a cytotoxic effect. The finding of *in vivo* bound complement in the lesions of systemic L.E. and "nephritis" is also consistent with the idea that *in vivo* complement binding is a factor involved in producing the low serum complement levels often found during the active stage of these diseases (30, 31).

In contrast, the great majority of plasma cells found in the tissues stained only with anti-7S γ-globulin or anti-19S γ-globulin and thus seem to contain unaggregated γ-globulin. However, in two tissues a few cells not readily distinguishable from plasma cells, stained with anti-β_{1C}. In both cases this was found in the spleen and in conditions (systemic L.E. and idiopathic thrombocytopenic purpura with hemolytic anemia) where a high level of complement production could reasonably be expected. It seems possible that these cells are producing the β_{1C} and this would be in accord with the work of Hochwald,

Thorbecke, and Asofsky (32) who have shown by a combination of immunoelectrophoreasis and amino acid incorporation studies that β_{1C}-globulin is formed in spleen and bone marrow.

The renal casts represent structures that are presumably not of immune origin. They showed staining with anti-7S γ-globulin and anti-19S as well as by the indirect rheumatoid factor method. Untreated they did not stain with anti-β_{1C}; after incubation with complement they did so to a variable degree. It would therefore appear that they contain some γ-globulin in aggregated form. However, since complement binding was shown only *in vitro*, this could not be implicated in any tubule damage.

In amyloid the picture was not uniform. In one case there was β_{1C} and no γ-globulin demonstrable and the indirect rheumatoid factor staining was weak. However, in the other two cases β_{1C}- and γ-globulin staining was parallel. In all three cases the β_{1C} was fixed *in vivo* so that it may have played some part in the disease.

In a number of individuals with miscellaneous conditions, results have been obtained whose relevance to the diseases concerned remains incomplete. However, it is of interest that four lesions in this limited survey stained with anti-β_{1C} after incubation with serum but not before. It is thus clear that *in vivo* fixation of C′ and the ability to fix C′ *in vitro* cannot be regarded as equivalent.

Why lesions that fix C′ *in vitro* do not do so *in vivo* is puzzling and a number of possibilities may be considered. It may be that in these cases the C′ fixation is an artefact; the treatment of the tissue during freezing and fixation producing the C′ binding sites in certain circumstances perhaps in the presence of large amounts of γ-globulin. However, it was not possible to produce C′ binding *in vitro* by freezing and thawing tissue in the presence of γ-globulin. A further cause of artefact could conceivably be the presence of unsuspected tissue-reactive antibodies in the C′ source. This probably can be excluded since anti-7S γ-globulin failed to stain, for example, the lymph node reticulum (T) after incubation with C′. The possibility that complement was in fact fixed *in vivo*, but that the β_{1C}-globulin had lost its antigenic determinants, could not be ruled out but it is difficult to see why, even in this case, the binding site should fail to fix β_{1C} *in vivo* when it can still do so *in vitro*. Probably an explanation of this effect must await more detailed information as to the conditions governing the *in vivo* fixation of complement. The reverse question—whether all sites that bind complement *in vivo* will also fix complement *in vitro*—has not so far been investigated.

It can be concluded that the detection of *in vivo* bound complement provides a further parameter by which the nature of lesions can be studied and that its spectrum is not identical with that provided by the detection of γ-globulin or by *in vitro* complement fixation. It seems likely that the *in vivo*

complement binding reflects processes at the binding site that, at least in part, are involved in the origin of the lesions there, and thus implicates some immune mechanism in their pathogenesis.

SUMMARY

A technique has been described for the demonstration of a human complement component by an immunofluorescent method. The component detected is β_{1C}-globulin, a moiety of the third complement component, which has previously been obtained in pure form and to which a specific antiserum has been prepared.

It has been shown in a model system that the binding of β_{1C}-globulin as shown by immunofluorescence is strictly equivalent to complement fixation as assessed by standard serological methods.

This technique has been applied to the detection of *in vivo* bound complement in pathological human tissues. It was found that *in vivo* complement binding occurs in the lesions of several human diseases, but not elsewhere in the same tissues. In a rather limited survey of diseases that has been carried out, *in vivo* complement binding was found particularly in systemic L.E., various nephritides, and amyloidosis, as well as in single cases of some other diseases.

The spectrum of *in vivo* complement binding has been compared with that of γ-globulin binding (7S and 19S types) and with the demonstration of *in vitro* complement fixation and rheumatoid factor fixation. It was distinct from each of these. Rheumatoid factor fixation, detected by anti-19S antiserum showed promise as a method for the detection of antigen-antibody complexes and aggregated γ-globulin in tissue sections.

The interpretation of these findings in regard to the nature of the binding sites, and their possible significance in regard to pathogenic mechanisms have been discussed.

The authors are greatly indebted to Dr. C. K. Osterland for performing many of the biopsies; and to Miss M. A. Calcott for technical assistance.

BIBLIOGRAPHY

1. Mellors, E. C., Arias - Stella, J., Siegel, M., and Pressman, D., Analytical pathology. II. Histopathologic demonstration of glomerular-localizing antibodies in experimental glomerulonephritis, *Am. J. Path.*, 1955, **31**, 687.
2. Dixon, F. J., Vazquez, J. J., Weigle, W. O., and Cochrane, C. G., Pathogenesis of serum sickness, *Arch. Path.*, 1958, **65**, 18.
3. Mellors, R. C., and Ortega, L. G., Analytical pathology. III. New observations on the pathogenesis of glomerulonephritis, lipid nephrosis, periarteritis nodosa and secondary amyloidosis in man, *Am. J. Path.*, 1956, **32**, 455.
4. Vazquez, J. J., and Dixon, F. J., Immunohistochemical analysis of amyloid by the fluorescent technique, *J. Exp. Med.*, 1956, **104**, 727.
5. Vazquez, J. J., and Dixon, F. J., Immunohistochemical study of lesions in rheu-

matic fever, systemic lupus erythematosus, and rheumatoid arthritis, *Lab. Inv.*, 1957, **6**, 205.

6. Mellors, R. C., Ortega, L. G., and Holman, H. R., Role of gamma globulins in pathogenesis of renal lesions in systemic lupus erythematosus and chronic membranous glomerulonephritis, with an observation on the lupus erythematosus cell reaction, *J. Exp. Med.*, 1957, **106**, 191.
7. Kaplan, M. H., and Dallenbach, F. D., Immunologic studies of heart tissue. III. Occurrence of bound gamma globulin in auricular appendages from rheumatic hearts. Relationship to certain histopathologic features of rheumatic heart disease, *J. Exp. Med.*, 1961, **113**, 1.
8. Vazquez, J. J., and Dixon, F. J., Immunohistochemical analysis of lesions associated with "fibrinoid change," *Arch. Path.*, 1958, **66**, 504.
9. Freedman, P., Peters, J. H., and Kark, R. M., Localization of gamma globulin in the diseased kidney, *Arch. Int. Med.*, 1960, **105**, 524.
10. Ross, A., and Lepow, I. H., Studies on immune cellular injury. I. Cytotoxic effects of antibody and complement, *J. Exp. Med.*, 1960, **112**, 1085.
11. (*a*) Müller-Eberhard, H. J., Nilsson, U., and Aronsson, T., Isolation and characterization of two β_1-glycoproteins of human serum, *J. Exp. Med.*, 1961, **111**, 201. (*b*) Müller-Eberhard, H. J., and Nilsson, U., Relation of a β_1-glycoprotein of human serum to the complement system, *J. Exp. Med.*, 1961, **111**, 217. (*c*) Müller-Eberhard, H. J., Isolation and description of proteins related to the human complement system, *Acta. Soc. Med. Upsaliensis*, 1961, **66**, 152
12. Goldwasser, R. A., and Shepard, C. C., Staining of complement and modifications of fluorescent antibody procedures, *J. Immunol.*, 1958, **80**, 122.
13. Klein, P., and Burkholder, P., Ein Verfahren zur fluoreszenzoptischen Darstellung der Komplementbindung und seine Anwendung zur histo-immunologischen Untersuchung der experimentellen Nierenanaphylaxie, *Deutsch. Med. Woch.*, 1959, **84**, 2001.
14. Vogt, A., and Kochem, H. G., Histo-serologische Untersuchungen mit fluoresceinmarkiertem Antikomplement, *Z. Zellforsch. u. Mikroskop. Anat.*, 1960, **52**, 640.
15. Strauss, A. J. L., Seegal, B. C., Hsu, K. C., Burkholder, P. M., Nastuk, W. L. and Osserman, K. E., Immunofluorescence demonstration of a muscle binding, complement fixing serum globulin fraction in myasthenia gravis, *Proc. Soc. Exp. Biol. and Med.*, 1960, **105**, 184.
16. Freedman, P., and Markowitz, A. S., Immunological studies in nephritis, *Lancet*, 1959, **2**, 45.
17. Burkholder, P. M., Littell, A. H., and Klein, P. G., Sectioning at room temperature of unfixed tissue, frozen in a gelatin matrix, for immunohistologic procedures, *Stain Technol.*, 1961, **36**, 89.
18. Marshall, J. D., Eveland, W. C., and Smith, C. W., Superiority of fluorescein isothiocyanate (Riggs) for fluorescent-antibody technic with a modification of its application, *Proc. Soc. Exp. Biol. and Med.*, 1958, **98**, 898.
19. Müller-Eberhard, H. J., and Kunkel, H. G., Isolation of a thermolabile serum protein which precipitates γ-globulin and participates in immune haemolysis, *Proc. Soc. Exp. Biol. and Med.*, 1961, **106**, 291.
20. Mayer, M. M., Procedure for titration of complement components with R1, R2,

R3, and R4, *in* Experimental Immunochemistry, (E. A. Kabat and M. M. Mayer, editors), Springfield, Illinois, C. C. Thomas, 2nd edition, 1961.

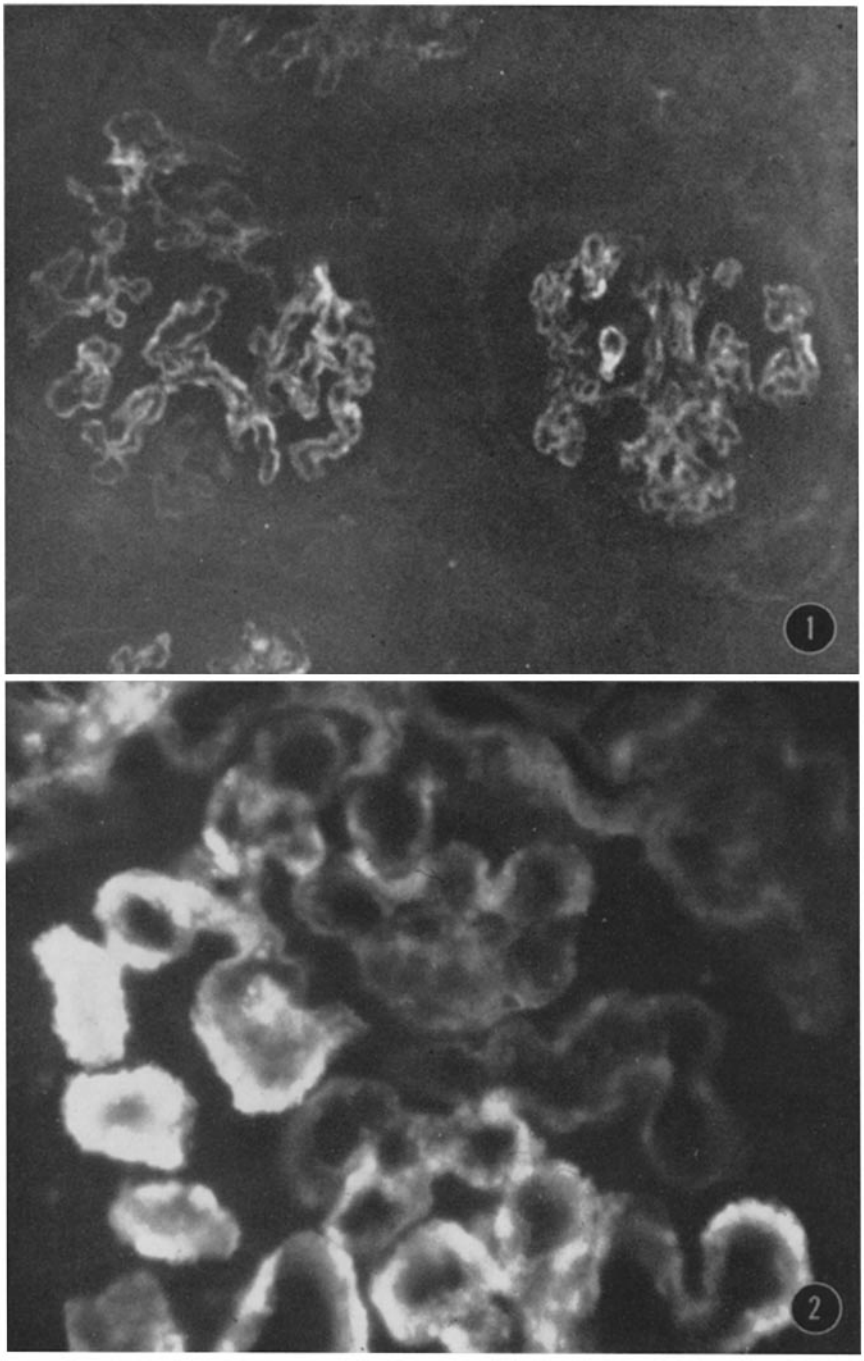
21. Ishizaka, T., and Ishizaka, K., Biological activities of aggregated gamma globulin. I. Skin reactive and complement-fixing properties of heat denatured gamma globulin, *Proc. Soc. Exp. Biol. and Med.*, 1959, **101,** 845.
22. Christian, C. L., Studies of aggregated γ-globulin. I. Sedimentation, electrophoretic and anti-complementary properties, *J. Immunol.*, 1960, **84,** 112.
23. Ishizaka, K. and Ishizaka, T., Biologic activity of aggregated γ-globulin. II. A study of various methods for aggregation and species differences, *J. Immunol.*, 1960, **85,** 163.
24. Franklin, E. C., Holman, H. R., Müller-Eberhard, H. J., and Kunkel, H. G., An unusual protein component of high molecular weight in the serum of certain patients with rheumatoid arthritis, *J. Exp. Med.*, 1957, **105,** 425.
25. Edelman, G. M., Kunkel, H. G., and Franklin, E. C., Interaction of the rheumatoid factor with antigen-antibody complexes and aggregated gamma globulin, *J. Exp. Med.*, 1958, **108,** 105.
26. Turk, J. L., Immune-adherence with soluble antigens, *Immunology*, 1958, **1,** 305.
27. Lachmann, P. J., unpublished observations.
28. Kunkel, H. G., The rheumatoid factors, *Arch. Int. Med.*, 1959, **104,** 832.
29. Christian, C. L., Studies of aggregated γ-globulin. II. Effect *in vivo*, *J. Immunol.*, 1960, **84,** 117.
30. Elliott, J. A., Studies on serum complement in disseminated lupus erythematosus, Thesis, University of Minnesota Graduate School, 1950.
31. Gunn, W. C., The variation in the amount of complement in the blood in some acute infectious diseases and its relation to the clinical features, *J. Path. Bact.*, 1914–14, **19,** 156.
32. Hochwald, G. M., Thorbecke, G. J., and Asofsky, R., Sites of formation of immune globulins and a component of C'3. I. A new technique for the demonstration of the synthesis of individual serum proteins by tissues *in vitro*, *J. Exp. Med.*, 1961, **114,** 459.
33. Scheidegger, J. J., Une micro-methode de l'immuno-electrophorese, *Internat. Arch. Allergy*, 1955, **7,** 103.

EXPLANATION OF PLATES

PLATE 8

FIG. 1. Glomeruli, lupus nephritis (patient A) stained with anti-β_{1C}. × 100.

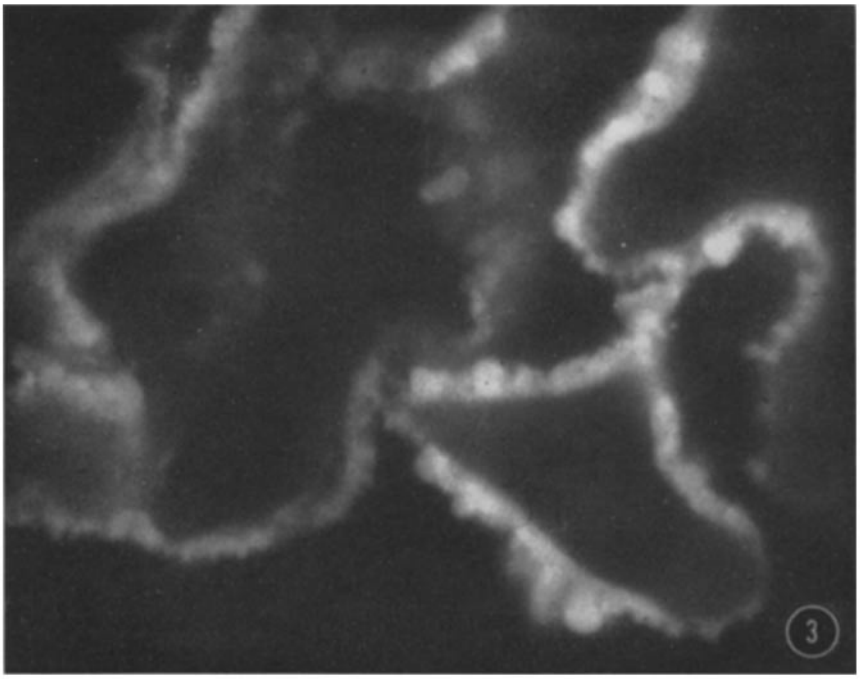
FIG. 2. Glomerular tuft, lupus nephritis (patient A) stained with anti-β_{1C} and at magnification 450 to show the basement membrane localization of the staining.



(Lachmann *et al.*: Complement in tissue sections)

PLATE 9

FIG. 3. Glomerular tuft, lupus nephritis (patient A) stained with anti-β_{1c} and at magnification 900 to show the basement membrane localization of the staining.

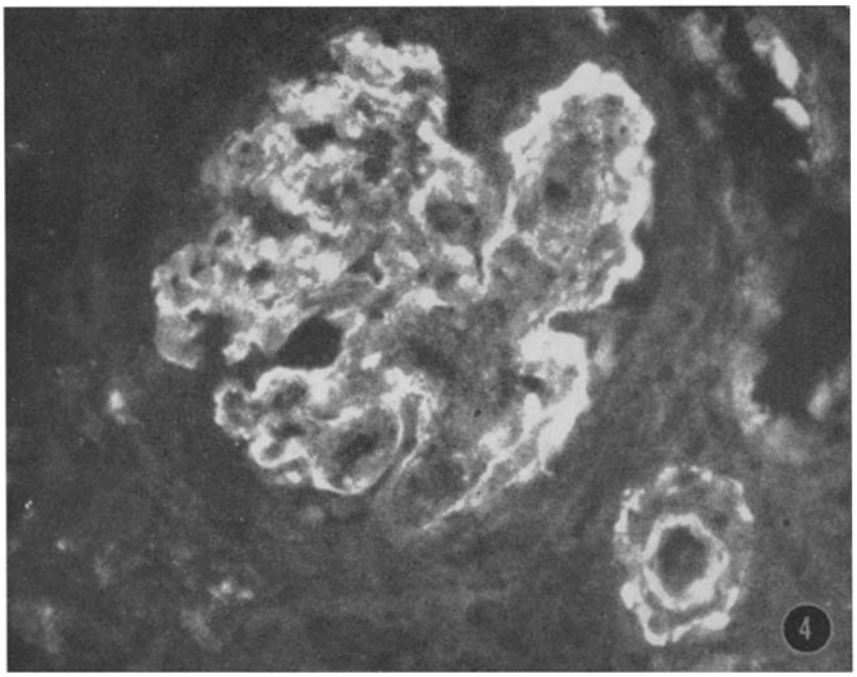


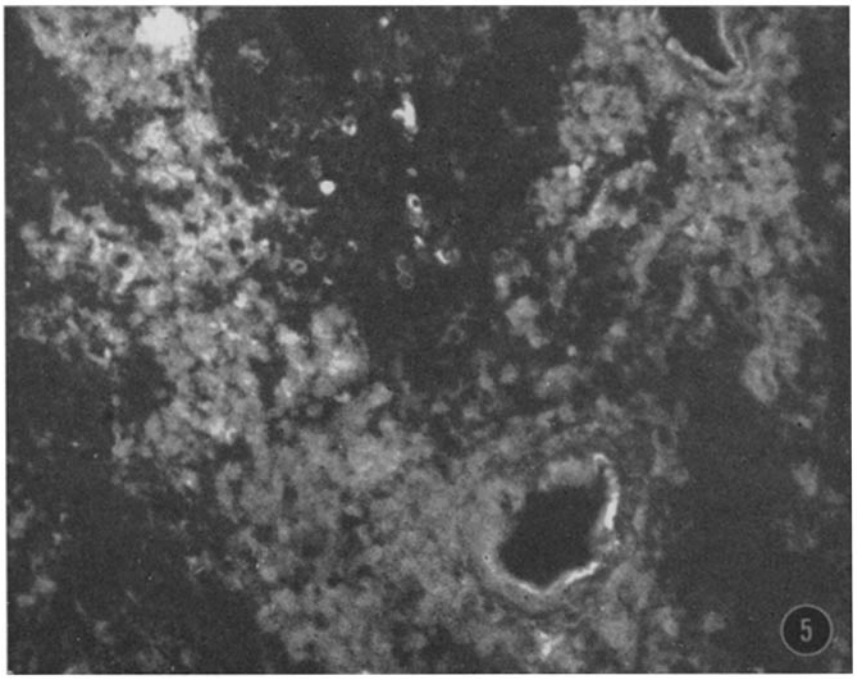
(Lachmann *et al.*: Complement in tissue sections)

PLATE 10

FIG. 4. Glomerulus and small vessel, malignant nephrosclerosis (patient N) stained with anti-β_{1C}. × 200.

FIG. 5. Amyloid deposits in the spleen (patient P) stained with anti-β_{1C} × 200.



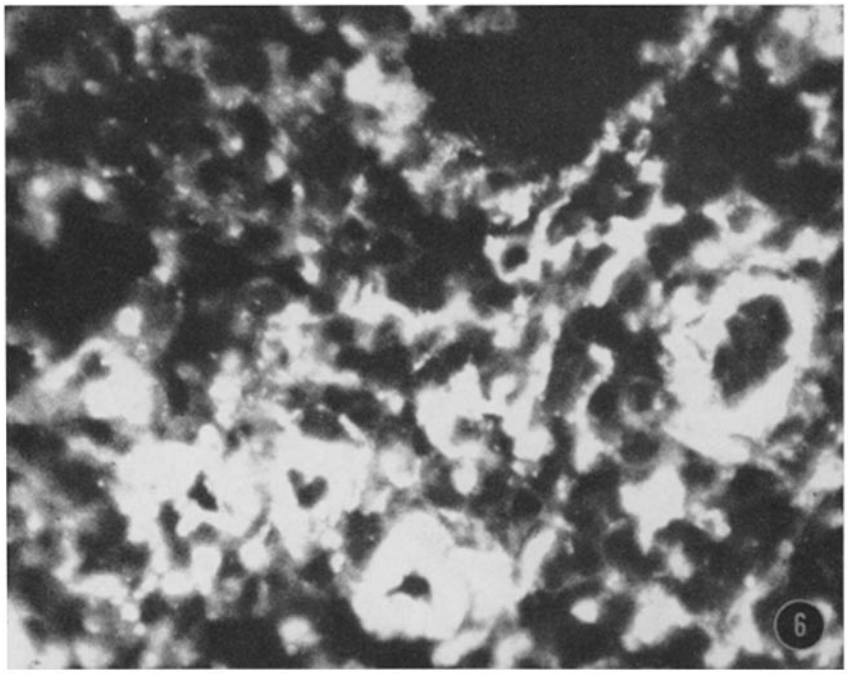


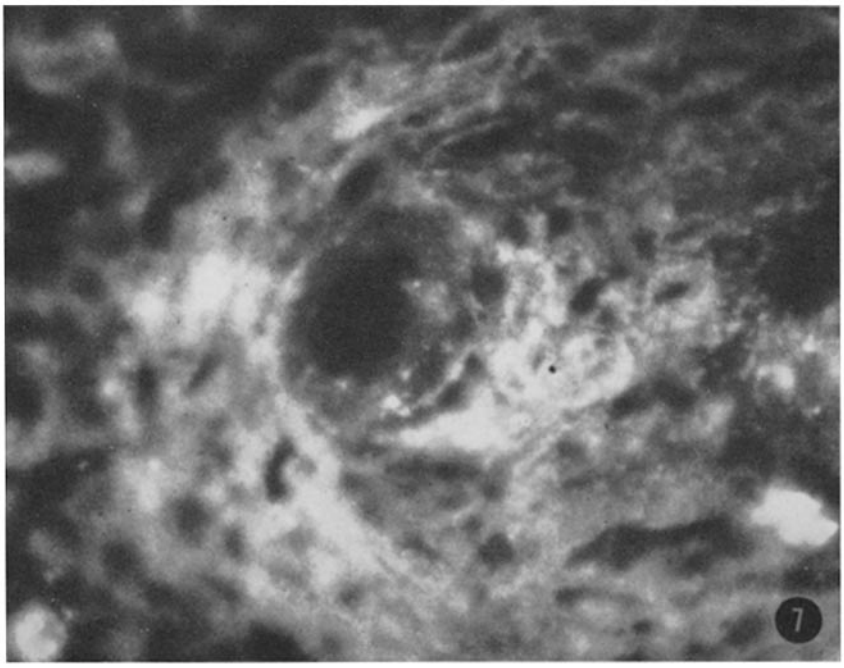
(Lachmann *et al.*: Complement in tissue sections)

PLATE 11

FIG. 6. Lymph node, allergic angiitis (patient T) stained with anti-β_{1C} after incubation with C′ to show reticulum staining. × 450.

FIG. 7. Lymph node, allergic angiitis (patient T) stained by indirect rheumatoid actor method to show reticulum staining. × 450.





(Lachmann *et al.*: Complement in tissue sections)