Importance of Immune Complex in Transplantation

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ABSTRACT: In the present study the role of antigen-antitissue antibody complex in causing tissue damage leading to systemic disease has been investigated using immunohistological methods. The membrane structure of microsomal fraction is considered immunologically quite complex i.e., consisting of a number of antigenically different molecules and hence only few of those molecules can be histocompatible and tissue specific.

Immune complex nephritis, amyloid deposition and systemic vasculitis up to fibrinoid degeneration of small arteries were successfully induced by prolonged sensitization with immune complex prepared by using a soluble microsomal fraction of lungs from allogenic mice as antigen. The similar changes but in lesser degree and frequency could be induced in mice when injected with antigen in high concentration with Freund's complete adjuvant in a prolonged period. This suggests that immune complexes formed from the subcellular component in the graft during a course of its decaying process can cause severe tissue damage in the recipient after transplantation.

KEY WORDS: immune complex, subcellular component, transplantation, decaying graft, amyloid deposition, systemic vascularitis, glomerulonephritis.

INTRODUCTION

Both cellular and humoral antibodies take part in the rejection mechanism in transplantation - the former acts mainly in the acute rejection and the latter mainly in the chronic. In either cases the graft acts as the source of antigens inducing various immunological and biochemical changes in the host body. This process is comparable to that of experimental sensitizaiton with tissue extract in a prolonged period.

In the present study the role of immune complex in transplantation has been investigated using immunohistological methods.

METHODS

Male dd mice of ca. 20 g in weight, averaging 4 weeks old, were used throughout the experiment.

Following apparata and reagents were used for preparation of materials. Sonicator (KMS-100, Kubota, Tokyo, Japan), Immunoelectrophoresis apparatus (PS-104-N, Kayagaki, Tokyo, Japan), Spectrophotometer (PMQII, Carl Zeiss, Oberkochen, West

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Germany), Freund's complete adjuvant (Difco Laboratories, Michigan, U.S.A.), Sephadex G-200 and G-50 (Pharmacia. A.B., Uppsala, Sweden), Diethylaminoethyl (DEAE)-cellulose, capacity 0.85 mgEq/g (Brown Co., New Hampshire, U.S.A.), Fluorescein isothiocyanate (FITC) isomer I (Baltimore Biological Laboratories, Maryland, U.S.A.).

Fluorescent microscopic examination was done with an ultraviolet microscope (Standard, Carl Zeiss, Oberkochen, West Germany) with a dark field condenser using a UV exciter filter. The light source was an Osram HBO-200 mercury arc lamp.

Antigen: Preparation of a soluble microsomal fraction from allogeneic lung (sMic-L); The lungs were removed from 100 mice after bleeding by postorbital puncture, stored immediately at −20°C and washed with cold 0.25M sucrose solution carefully after thawing and cut into small pieces with scissors. Four times as much volume of cold 0.25 M sucrose solution was added and homogenized with a Potter-Elvehjem homogenizer. Centrifugation at 13,000 g for 30 min. removed whole cells, debris, nuclei and mitochondria. Subsequently the microsomal fraction was obtained by centrifugation of the supernatant at 105,000 g for 60 min. Finally the sediment was suspended in 0.25 M sucrose solution and sonicated at 20 KC/sec for 15 min. The sMic-L was prepared from the supernatant after centrifugation at 13,000 g for 1 hour. All process was carried out at 4°C.

Preparation of immune complex using sMic-L; 2.5 ml of sMic-L emulsified with an equal volume of Freund's complete adjuvant was injected into 50 mice on the back subcutaneously once a week for three months. Protein content of each dose was adjusted to 200 μgN/ml. After completion of sensitization mice sera were obtained by bleeding from the tail veins and incubated with optimal amount of antigen (referring to the precipitin curve estimated beforehand) for 2 hours at 37°C and centrifuged at 500 g for 30 min. at 4°C. The supernatant was discarded, an equal volume of saline was added and centrifuged again. This process was repeated three times. The precipitate (insoluble immune complex) was finally suspended in saline.

Inoculation of animals: The mice were divided into three groups. In group I, 2 ml of insoluble immune complex using sMic-L was injected into 20 mice on the back subcutaneously once a week for three months. Protein content of complex was found to be 80–100 μgN/ml. 2 ml of sMic-L with an equal volume of Freund's complete adjuvant was injected repeatedly into 20 mice of group II in order to produce immune complex in vivo. Inoculation procedure was same as described above. Protein content was adjusted to 200 μgN/ml. Group III was served as control since spontaneous amyloid deposition was reported previously.[15]

Preparation of labeled antibody: Preparation of mouse IgG; A crude globulin fraction of normal mouse serum obtained by three times of precipitation with 34 per cent saturated ammonium sulphate (SAS) was subjected to gel filtration on a Sephadex G-200 column using phosphate-buffered saline (PBS), pH 7.2, as an eluting buffer. The fractionation of eluate yielded two major peaks. The descending part of the second peak was concentrated with the use of polyvinylpyrrolidone (PVP) and the concentrate was applied on a DEAE-cellulose column equilibrated with 0.01 M phosphate buffer (PB), pH 8.6. Stepwise elution was made as indicated in Fig. 1. The ascending slope of the first peak, eluted with 0.01 M PB, pH 8.4, containing IgG was concentrated by PVP and dialysed against PBS, pH 7.2. Chromatographically purified IgG revealed a single precipitin band in the γ-region by immunoelectrophoresis against rabbit anti-mouse

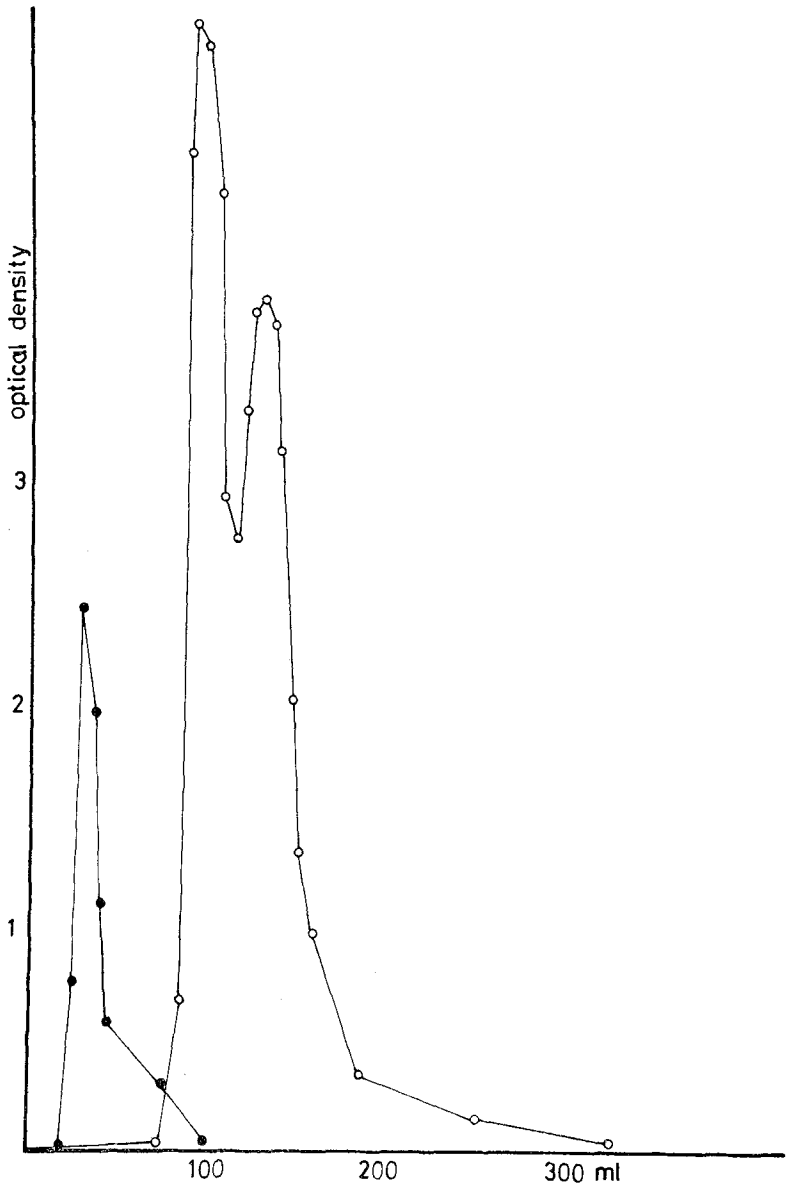

Fig. 1.—Preparation of mouse IgG
○——○ mouse crude globulin on Sephadex G-200 column. Flow rate 6 ml/hr, Gel bed 900× 25 mm, Eluting Solution PBS, pH 7.2, 0.01 M.
●——● DEAE-cellulose column chromatography of filtrated crude globulin (stepwise elution). Only fraction-I was described.

whole serum.

Preparation of anti-mouse IgG rabbit serum; 2 ml of mouse IgG (5 mg/ml) were emulsified with an equal volume of Freund's complete adjuvant and multiple injections were made subcutaneously in two male albino rabbits. The rabbits received a booster injection after three weeks and were bled one week after the booster after confirming im-

munoelectrophoresis showing single γ-line against mouse whole serum. The contents of specific antibody in 1 ml of the antisera obtained were estimated from a precipitin curve using a modified micro-Kjeldahl method and found to have 2,490 and 1,580 μgN/ml.

Preparation of anti-sMic-L rabbit serum; sMic-L was used for immunization and the method of preparation was same as those used in preparation of anti-mouse IgG rabbit serum. Antisera were obtained one week after the booster. One tenth volume of mouse blood was added in order to absorb the component of mouse blood. Passive hemagglutinin test using glutaraldehyde as coupling reagent[1] showed 3,200 in titer.

Preparation of conjugate; Crude globulin of anti-mouse IgG rabbit serum was prepared by applying salting out procedure with SAS three times, diluting to a final protein concentartion of 10 mg/ml and adjusting to pH 9.1 with 0.5 M sodium carbonate bicarbonate buffer. FITC was added and stirred at 4°C for six hours. Fluorescein to protein weight ratio was 1 : 60. Free fluorochrome was removed by gel filtration on Sephadex G-50. Unlabeled and overlabeled protein were eliminated by purification with DEAE-cellulose column chromatography. Stepwise elution according to Sober and Peterson[18] resulted in four fractions with increasing fluorescein to protein molar ratio. The protein content of each fraction was measured by micro-Kjeldahl method and their absorbances at 280 mμ and 495 mμ were read with a spectrophotometer. Fluorescein to protein molar ratio was then determined. Only Fx-1 fraction eluted with 0.05 M PB, pH 6.4 was used throughout the investigation. The conjugate was sterilized by passing through a millipore filter (Type HA) and stored at −20°C.

The same procedure was employed for the labeling of FITC with anti-sMic-L rabbit γ-globulin.

Tissues: The lungs, kidneys, liver and spleen removed were fixed with 95 per cent cold ethanol and embedded in paraffin.[8,17] Sections for light microscopy were stained with haematoxylin-eosin, Congo red and periodic acid-Schiff stain. Sections for fluorescent microscopy were incubated with each conjugate in the moist chamber overnight, washed with staining buffer and mounted with glycerol buffer, pH 9.5. Sections for detection of amyloid deposits were stained with alum-haematoxylin for 5 min. to quench the nuclear autofluorescence, dipped in one per cent Thioflavin-T for 10 min., washed with running water for 20 min., differentiated with one per cent acetic acid and mounted with glycerol buffer, pH 7.2.

Results

The finding of rescipient's lung was described at first since the antigen was prepared from lungs of allogeneic mice. In group I, there were congestion in lung vessels and occasional presence of macrophages or histocytes in the alveolar spaces. Excessive collection of lymphoid cells around the thickened arteries and of plasma cells under the subbronchial layer, supposed to be coming from proliferated lymph apparata or lymph nodes, in which germinal centers showed active response, was prominent findings.

Anti-mouse IgG conjugate revealed remarkable proliferation of plasma cells around bronchi and bronchioles, containing marked amount of antibody (Fig. 2). Anti-sMic-L conjugate stained some parts of the alveolar cell membrane and that of macrophages or histiocytes. Specific fluorescence by these two conjugates could not be observed at the same part in the lung.

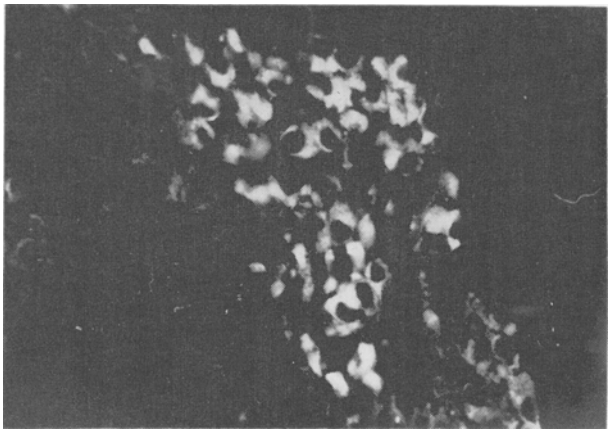

Fig. 2.—Fluorescent micrograph of the lung after injection of immune complex. Note the collection of plasma cells stained with FITC labeled anti-mouse IgG conjugate. (500 ×)

Group II showed similar findings but in lesser degree in all cases. No abnormal changes in group III.

1) *Immune complex nephritis* In all cases of group I, light microscopy revealed more or less variable changes ranging from enlargement of glomeruli, increased or decreased number of mesangial cells, occupation of mesangial matrix by amorphous acellular mass, glomerular adhesion to the capsule and to capsular crescent formation. Some tubules were dilated and contained eosinophilic casts and their epithelial cells were found to be turbid and swollen.

Under fluorescent microscopy specific fluorescence was found along the glomerular basement membrane in a granular or sometimes in the form of clumps. Antigen, proved by anti-sMic-L conjugate, showed focal distribution at the glomeruli, compared with relatively diffuse pattern of antibody detected by anti-mouse IgG conjugate (Fig. 3). Fluorescent intensity of antigen or antibody was strong enough to be distinguished from that found at the portion of amyloid accumulation. This glomerular lesion was supposed to be induced by immune complex due to the coexistence of antigen and antibody at the same parts of glomeruli. Sometimes tubular epithelial cells were stained with anti-mouse IgG conjugate. Its fluorescence may be due to resorption of leaked protein or epithelial cell necrosis.

Immune complex nephritis was observed in 9 cases having amyloid deposit in group II and none in group III.

Table 1.—Frequency of lesions caused by immune complex (number of pathological case/ total number)

	Immune complex nephritis	Amyloid deposition			Acellular Vasculitis
		Spleen	Kidney	Liver	
Group I	14/14	14/14	14/14	14/14	14/14
Group II	9/13	9/13	8/13	8/13	10/13
Group III	0/10	0/10			0/10

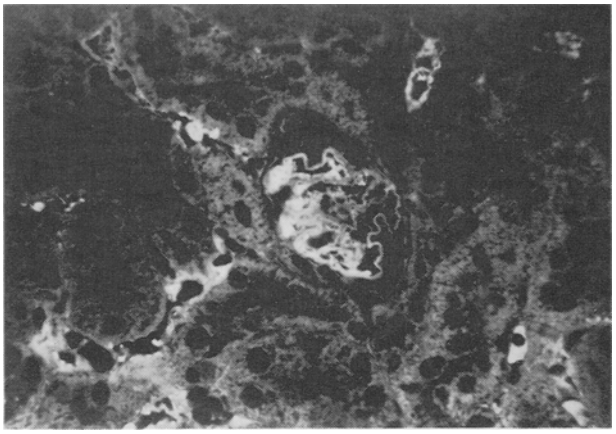

Fig. 3.—Specific fluorescence found along the glomerular basement membrane stained with FITC labeled anti-mouse IgG conjugate. (200 ×)

2) *Amyloid deposition* In the spleen, amorphous acellular eosinophilic masses were seen in all cases (14) of group I and in 9 out of 13 animals in group II, but in group III such substance could not be found at all. This mass showed a similar character to human amyloid, i.e., weakly positive with Congo red, relatively positive with periodic acid-Schiff and giving yellow fluorescence with Thioflavin-T. This substance was first seen at the marginal zone of the follicles and the surrounding of vessels in 4 cases of group I and 5 in group II. In 2 cases of group I and group II, the mass extended to the red pulp, which was almost replaced by the mass in 8 cases of group I. Splenocytes were not invaded even in severe cases.

The deposit of amyloid was stained positive with both anti-mouse IgG conjugate and anti-sMic-L conjugate, but its fluorescent intensity was much less than that in the cytoplasm of plasma cells which was compressed by the deposit of amyloid to a variable degree. There was a marked proliferation of plasma cells, active germinal response and lightly stained reticular cells with periodic acid-Schiff in the cases with no amyloid

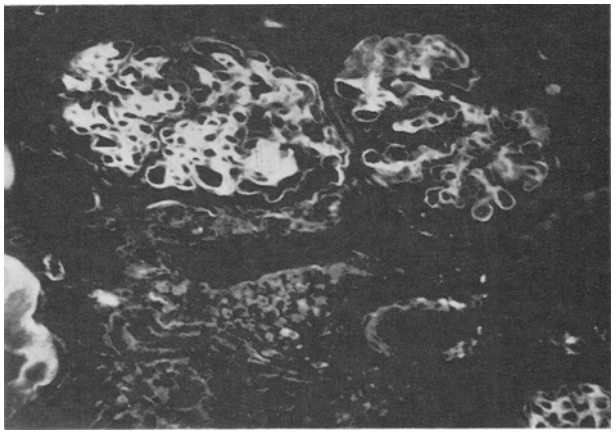

Fig. 4.—Bright yellow fluorescence at the mesangial matrix and tubular cast stained with Thioflavin-T. (500 ×)

deposition in group II. The spleen was the first organ in which amyloid deposition was found.

In the kidney amyloid deposition could be found in the mesangial region of the glomerular tuft in all cases of group I and 8 in group II (Fig. 4). This mass showed the same staining characteristics as mentioned above. In severe cases, mostly observed in group I, the number of mesangial cells decreased and glomerular capillaries were entirely compressed. In the interstitium between the collecting tubuli, the same mass was found accompanied by cell infiltration of a marked degree, mainly of the lymphoid cells and plasma cells and compression to the adjacent structure by this mass was observed. The cast in the tubules was also stained with anti-mouse IgG conjugate and showed fluorescence with Thioflavin-T.

The liver in all of group I and 8 cases in the group II showed the amyloid deposition in the vascular endothelial linings of hepatic and portal veins and Disse's space in the peripheral region of the liver acini. Abundant deposits of amyloid tend to cause pressure atrophy of parenchymatous cells and the normal architecture of acini was not found in 8 cases of group I. This mass and plasma cells collected at the Glisson's sheath showed fluorescence when stained with anti-mouse IgG conjugate, but difference in staining intensity was similar to that found at the glomerular mesangium.

In the lung no accumulation of amyloid was observed in all groups.

3) *Acellular vasculitis* In group I, thickened wall of small artery, homogenously stained eosinophilic with haematoxylin-eosin, was observed in the liver, spleen and lung. This fibrinoid degeneration was thought to be the deposit of some unknown protein, though a few of thickened small arteries showed fluorescence with anti-mouse IgG conjugate and anti-sMic-L conjugate (Fig. 5). These abnormal small arteries were always accompanied by a marked collection of lymphoid and plasma cells. The latter showed specific fluorescence with anti-mouse IgG conjugate.

These changes being variable in severity were also found in 10 of group II and none in group III.

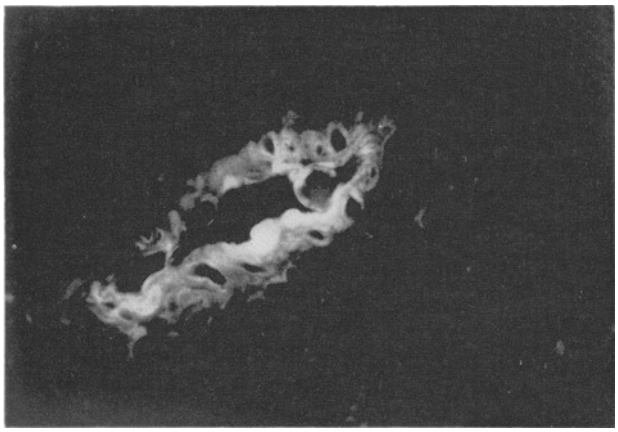

Fig. 5.—The vessel in the spleen stained with FITC labeled anti-sMic-L conjugate. (500 ×)

Discussion

Hume[11] suggested two sites involved in chronic rejections, where the humoral antibody plays the main role, i.e., at the endothelium of the renal vasculature and at the glomerular basement membrane. It is postulated that these lesions may be caused by direct attack of antibodies or by subsequent changes such as autoimmune disease, immune complex disease or various biochemical changes provoked by antibodies or antigen antibody reactions.

This investigation was designed using allogenic antitissue antibody to study the behavior of insoluble immune complexes when introduced into animals in high concentration and to find the role of immune complex in the transplanted recipient. A soluble microsomal fraction of lungs from allogenic mice containing various kinds of antigenic molecules, was used as an antigen. When introduced as insoluble complex, some part may separate into antigen and antibody or become soluble in the presence of excess antigen or antibody, but some may remain insoluble and subsequently be trapped in the vascular endothelium or carried to glomerulus directly thus causing severe damage, i.e., glomeruonephritis, amyloid deposition and systemic vasculitis up to fibrinoid degeneration of thickened arterioles surrounded by marked accumulation of lymphoid and plasma cells. Cellular collection is thought to express hyperimmune environment histologically as in a case of proliferated lymph apparata and active germinal response in lymph nodes found in the lung. Antigen and antibody (IgG) demonstrated at the same part by fluorescent antibody technique indicate location of immune complex. Small complexes may remain in circulation without being deposited in tissue while larger complexes seem to be trapped in tissue to cause proliferative endothelial lesion with necrosis. Hyaline degeneration occurs in this chronic state.[4] Fibrinoid necrosis of small hepatic arteries in the canine hepatic allografts[14] may thus be caused by immune complex. Benacerraf et al.[3] and McClusky et al.[13] demonstrated the etiologic role of complexes the in renal lesion by infusing soluble complexes into normal animal and producing endocarditis, arteritis and glomerulitis.

Amyloid deposits were observed in vascular endothelial linings of hepatic and portal veins and Disse's space in the liver, in red pulp and perifollicular zone in the spleen and in mesangial matrix and subendothelial region of glomerular capillary. Antigen and antibody (IgG) were also detected by fluorescent antibody technique in the present experiment. In laboratory animals, amyloidosis can be produced readily by various methods such as inoculation with casein,[6,19,20] albumin[9] and ribonucleate[10,16] or by graft-versus-host reaction,[5] parabiosis[24] and transfer of the spleen cells[21,22,23] in inbred mice. Demonstration of large amounts of both γ-globulin[20] and complement[12] at the site of amyloid deposits suggests that amyloid represents extravascular deposit of γ-globulin, immunoglobulin fragment or immune complex itself. Rescently[7] murine amyloid protein was identified as the NH_2-terminal variable fragment of a mouse immunoglobulin λ light chain by biochemical and immunochemical analysis. Azar[2] proposed that amyloid deposits represent a broad group of protein complex including γ-globulin or its fragment, which polymerizes extracellulary in the form of fine fibrils. Those changes of γ-globulin to paraprotein seem to be responsible for the difference in fluorescent intensity between amyloid and plasma cells. Similar lesions but in lesser degree were found in the mice injected with allogenic alone. Immune

complex, in general, can often be produced when antibody encounters with antigen in high concentration, but severe tissue damage does not develop because of the rapid excretion of immune complex in the early stage of hyperimmune environment. Several factors such as duration of sensitization, combination with complement, permeability of vessel, difference in molecular weight and antigenic nature are considered to be related to making immune complex remain in circulation and aggregate to become less soluble thus causing tissue damage. In man, immune complexes are likely to be produced from the subcellular component released from the decaying graft and may produce pathological changes in the body similar to those in mice found in the present investigation, though histological expression may be different.

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References

1. Avrameas, S., Taudou, B. and Chuilon, S.: Glutaraldehyde, cyanuric chroride and tetra-azotized o-dianisidine as coupling reagents in the passive hemagglutination test, Immunochemistry 6: 67–76, 1969.
2. Azar, H. A.: Pathology annual, pp. 105–122, Appleton-Century-Croft, New York, 1968.
3. Benacerraf, B., Potter, J. L., McCluskey, R. T. and Miller, F.: The pathologic effects of intravenously administered soluble antigen-antibody complexes. II. Acute glomerulonephritis in rats, J. Exp. Med. 111: 195–200, 1960.
4. Dixon, F. J., Feldman, J. D. and Vazquez, J. J.: Experimental glomerulonephritis. The pathogenesis of a laboratory model resembling the spectrum of human glomerulonephritis, J. Exp. Med. 113: 899–920, 1961.
5. Druet, R. L. and Janigan, D. T.: Experimental amyloidosis. Rates of induction, lymphocyte depletion and thymic atrophy, Amer. J. Path. 49: 911–929, 1966.
6. Giles, R. B. and Calkins, E.: The relationship of serum hexosamine a globulin and antibodies to experimental amyloidosis, J. Clin. Invest. 37: 846–857, 1958.
7. Glenner, G. G., Page, D., Isersky, C., Harada, M., Cuatrecasas, P., Eanes, E. D., DeLellis, R. A., Bladen, H. A. and Keiser, H. R.: Murine amyloid fibril protein: Isolation, purification and characterization, J. Histochem. Cytochem. 19: 16–28, 1971.
8. Hamashima, H. and Kyogoku, M.: Immunohistology, pp. 82, Igaku-Shoin, Tokyo, 1968 (in Japanese).
9. Heefner, W. A. and Sorenson, G. D.: Experimental amyloidosis Light and electron microscopic observations of spleen and lymph node, Lab. Invest. 11: 585–593, 1962.
10. Huestis, D. W. and Jaeger, E. A.: Studies on serum protein, glucoproteins and sialic acid in experimental amyloidosis, Arch. Path. 70: 166–170, 1960.
11. Hume, D. M.: Organ transplantation today, pp. 311–313, Excerpta Medica Foundation, Amsterdam, 1969.
12. Letterer, E., Caesar, R. and Vogt, A.: Studien zur elektronenoptischen und immunomorphologischen Struktur des Amyloids, Deutsch Med. Wschr. 85: 1909–1910, 1960.
13. McCluskey, R. T., Benacerraf, B., Potter, J. L. and Miller, F.: The pathologic effects of intravenously administered soluble antigen-antibody complexes. I. Passive serum sickeness in mice, J. Exp. Med. 111: 181–194, 1960.
14. Porter, K. A.: Pathology of liver transplantation, Transplantation Review 2: 129–170, 1969.
15. Ram, J. S., DeLellis, R. A. and Glenner, G.G.: Amyloid, Int. Arch. Allergy 35: 288–297, 1969.
16. Richter, G. W.: Alterations in serum globulins during the formation and resorption of amyloid in rabbits, J. Exp. Med. 104: 847–863, 1956.

17. Sainte-Marie, G.: A paraffin embedding technique for studies employing immunofluorescence, J. Histochem. Cytochem. 10: 250–256, 1962.
18. Sober, H. A. and Peterson, E. A.: Protein chromatography on ion exchange cellulose, Fed. Proc. 17: 1116–1125, 1958.
19. Sorenson, G. D. and Shimamura, T.: Experimental amyloidosis Light and electron microscopic observations of renal glomeruli, Lab. Invest. 13: 1409–1417, 1964.
20. Vazquez, J. J. and Dixon, F. J.: Immunohistochemical analysis of amyloid by the fluorescence technique, J. Exp. Med. 104: 727–747, 1956.
21. Werdelin, O. and Ranlφv, P.: Amyloidosis in mice produced by transplantation of spleen cells from casein-treated mice, Acta Path. Microbiol. Scand. 68: 1–18, 1966.
22. Werdelin, E.: Amyloidosis induced by cell transfer. Effects of heat-damaged and of x-irradiated spleen cells from casein-sensitized mice, Acta Path. Microbiol. Scand. 72: 23–30, 1968.
23. Werdelin, O.: Lung amyloidosis induced in mice by transplantation of casein sensitized spleen cells, Acta Path. Microbiol. Scand. 73: 60–72, 1968.
24. Williams, G.: Amyloidosis in parabiotic mice, J. Path. Bact. 88: 35–41, 1964.