Quantitative Comparative Immunohistology

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A quantitative assay for the determination of fluorescence of tissue sections stained with fluorescein-labeled antibodies using a fluorometer is described. Sequential microtome sections were stained with labeled antiserums, washed repeatedly with buffered saline, and digested with 20% (w/v) NaOH at 60° for 12 hr. The fluorescence of the resultant solution is then read in a fluorometer. There is similarity between sequential sections of the same biopsy specimen. Specific blocking procedures reduce the readings of the fluorescence markedly. By comparison with values obtained simultaneously from standard curves of the labeled antiserums used, the amount of bound antibody or specific proteins can be determined quantitatively.

Immunofluorescence technics as diagnostic tools and therapeutic guides in renal diseases have been widely employed (1, 2). γ-Globulin (IgG) and the βw component of complement can be visualized qualitatively on kidney biopsy specimens in the different stages of glomerulonephritis and other renal diseases caused by complement-binding antigen-antibody reactions (3). In the course of a study on the nature of the specific antigen in acute poststreptococcal glomerulonephritis, the availability of a sensitive quantitative comparative assay for the fluorescein-marked antigen-antibody complement complexes, especially after specific absorption procedures on the serum, was considered to be desirable. Such a method for the quantitation of immunofluorescent aggregates was, therefore, developed.

Method

Serums were fractionated with 6,9-diamino-2-ethoxyacridine (Ethodin), and the IgG fraction was conjugated with fluorescein isothiocyanate (4). The unreacted fluorescein was removed by gel filtration through Sephadex G25 columns. All labeled serums were adsorbed with homogenates of fresh rat liver in a proportion of 1:1.

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Fresh autopsy specimens or kidney biopsy specimens embedded in rat liver were quick frozen in a 2-methyl butane Dry Ice mixture (2). Sequential sections of 3 μ cut in a cryostat were placed individually in the bottom of 50-ml beakers. Other sections were placed on slides and examined under the microscope. Uniformity of the sections is most critical, and only identical sections were used. The sections in the beaker were first prewashed for 15 minutes in phosphate-buffered saline 0.01 M, pH 7.1, and the supernatant was decanted carefully. The specimens were then covered with 0.1 ml of fluoresceinated antiserum and placed in moist chambers for 30 min. Thereafter the sections were washed at least five times with phosphate-buffered saline until the final wash had a zero reading in the fluorometer (Turner fluorometer Model 110). To the beaker ten ml of 20% (w/v) NaOH was added, and the sections were digested at 60° for 12 hr. The resultant solution was appropriately diluted, if necessary, with 20% NaOH to fall into the fluorometer's range, and then read.

**Results**

Fluorometric readings of the specimens were compared with standard curves of the values obtained from different dilutions of the fluoresceinated antiserum identically treated. Figure 1 represents a typical curve of fluorescein-labeled rabbit anti-human IgG. The degree of protein-fluorescein conjugation obtained varied slightly from batch to batch; therefore, studies were performed with single batches stored in small aliquots at -20°. New standard curves were prepared each time from the frozen samples used for the tissue staining, since, even under freezing, uncoupling of fluorescein from the protein may occur.

**Fig 1.** Fluorescence of different concentrations of fluorescein-labeled rabbit anti-human IgG in micrograms per 100 ml. Solutions were diluted in 20% NaOH and incubated at 60° for 12 hr.
Heat denaturation and protein hydrolysis did not affect the absorption spectrum, confirming the results of others (5). Linearity was retained under our conditions; similarly, quenching and light scattering had no effect. Higher fluorescence readings of the samples and the standards can be obtained when the specimens are brought to pH 7.0. This, however, caused complications—ie, large dilutions with buffers or addition of concentrated acids to the 20% NaOH. This modification, therefore, was not used.

In their early phase of acute poststreptococcal glomerulonephritis, 6 patients showed the usual localization of IgG and complement (βc-βd) on the basement membranes and in the mesangium of the glomeruli. Table 1 gives the results of the quantitative readings in the fluorometer obtained from a typical biopsy specimen. The unstained section had some reading, probably due to the well known autofluorescence of tissue proteins. The fluorescence of the sections stained with labeled anti-IgG and anti-complement, however, was approximately 12 times higher than the fluorescence obtained from the unstained section. There was, of course, wide variation in the amounts of IgG and complement fluorescence between specimens from different patients dependent on the number of glomeruli in the specimen, their size, and other individual variations. Within the same patient’s sections, however, there was an excellent agreement in the fluorometer reading. Fluoresceinated anti-IgG staining of 10 sequential sections gave readings with differences of less than ±6%. This was confirmed in six biopsy specimens. When unlabeled antiserums were first used for blocking purposes for 3 hr, and then the labeled fractions were added, the fluorometer readings were higher than the readings from the unstained sections, but much lower than the readings of sections stained directly without blocking. We feel that this was due to incomplete blocking. Blocking for 24 hr reduced the fluorescence to a level much closer to the control unstained sections (Table 1).

| Table 1. Amounts of Fluoresceinated Anti-IgG and Anti-βd on Renal Section from Patient with Acute Poststreptococcal Glomerulonephritis |
|---------------------------------|------------------|
| **Section**                     | µg/100 ml        |
| Control unstained section*      | 50.0             |
| Stained anti-βc                 | 700.0            |
| Stained anti-IgG                | 780.0            |
| Blocked 3 hr anti-IgG unlabeled† | 170.0            |
| Blocked 24 hr anti-IgG†         | 82.0             |

* Fluorescence reading of the unstained tissue section was due to native fluorescence of tissue proteins.
† Section blocked with unlabeled anti-IgG for time indicated and then stained with fluoresceinated anti-IgG.
Labeled IgG fractions from acute poststreptococcal glomerulonephritic patients stain their own glomeruli and those of the other nephritics interchangeably (e). These fractions were absorbed uniformly with various bacteria, including three known nephritogenic strains. Ultraviolet microscopic examinations qualitatively showed markedly less fluorescence with the IgG fractions absorbed with the nephritogenic strains than those of the other bacteria. The quantitative method confirmed this observation with a 70% reduction in the fluorescence of the fractions absorbed with nephritogenic streptococci compared to absorption with other bacteria which did not lead to any reduction in fluorescence.

Discussion

This quantitative assay thus provides a useful tool in conjunction with microscopic examinations. Quantitative data concerning the amounts of labeled antibodies of different kinds bound to a given tissue can be obtained providing the sections from the same biopsy specimen of a patient are compared, and a simultaneously determined curve of the fluorescence of the serum at different concentrations is available. The method is ideally suited in those instances where labeled serums are absorbed with various agents and the quantitative effect of the absorption is of importance. It should be emphasized, however, that quantitative results on specimens from different patients cannot be compared owing to the discrepancy in number and size of structures—eg, glomeruli involved in the immune process in sections from different biopsy specimens.

References