7. Pochyly, D. F., Federation Proc. (Abst.) 26, 478 (1967).
8. Bloom, B. R. and Bennett, B., Federation Proc. 27, 13 (1968).
9. Chefurka, W. and Haiasai, Y., Biochem. Biophys. Res. Commun. 24, 633 (1966).
10. Kaji, H., Suzuka, I., and Kaji, A., J. Mol. Biol. 18, 219 (1966).
11. Rabinovitch, M., Proc. Soc. Exptl. Biol. Med. 124, 396 (1967).

Received Sept. 19, 1968. P.S.E.B.M., 1969, Vol. 130.

# Interaction of Aggregates of Reduced Insulin with Gamma Globulin and Complement: A Pathogenetic Hypothesis* (33590)

ROBERT M. STROUD, JERALD W. CANTRELL,[1] AND KENNETH M. PRUITT
(introduced by J. M. McKibbin)

*Department of Biochemistry, Department of Medicine, and Laboratory of Molecular Biology, University of Alabama in Birmingham, Birmingham, Alabama 35233*

Berns *et al.* (1) showed that in some of the glomerular lesions found in diabetics deposits of gamma globulin and insulin are present. Burkholder (2) demonstrated that kidney slices from some diabetic patients were capable of fixing guinea pig complement *in vitro* as shown by the immunofluorescent antibody technique. Many studies of several varieties of experimental immunopathologic renal disease have shown that antigen–antibody complexes are found in glomeruli along with certain complement components (3). Involvement of the complement system seems to be required for the full expression of some of these experimental glomerular lesions. Studies of glomeruli from patients with systemic lupus erythematosus and acute glomerulonephritis (4) lend further support to the pathogenetic role of the complement system in renal damage.

These reports suggested to us the hypothesis that gamma globulin in association with insulin or insulin derivatives might be pathogenic and that insulin or some of its derivatives could produce renal and vascular lesions by a mechanism involving the complement system.

Insulin is degraded *in vivo* (5) and *in vitro* (6) by both reductive and proteolytic enzymes. This degradation has been observed in liver, kidney, adipose tissue, muscle, and the pancreas. A consistent feature of these various degradation reactions is the release of insulin A and B chains and their fragments. The biological activity of these degradation products in combination with other serum proteins has been a matter of controversy for several years (7). The data available at the present time seem to indicate that the A and B chains and their fragments have little or no insulin-like activity and no consistently demonstrable effect on the expression of insulin activity (8). However, it has been reported that the A and B chains do circulate *in vivo* (9). It also seems likely that immunoassayable insulin does not bind tightly to other serum proteins (10).

High levels of circulating, immunoassayable insulin are often associated with diabetes and/or obesity, and the combination of insulin fragments with other serum proteins substantially increases their half-life in plasma (11). However, at the present time there is no information on the structure and no quantitative data on the amounts of insulin derivatives which accumulate *in vivo*. Since diabetics frequently receive 1–2 mg of insulin daily, since the daily insulin secretion by the normal pancreas is of similar magnitude, and since insulin is continuously degraded, the ac-

* Supported in part by NIH grants AI 08422, AM 03555, and American Cancer Society Grant T-921 and PHS Grant AM 10863-02.

[1] National Defense Education Act Graduate Fellow.

cumulation of significant quantities of partially degraded insulin or individual chains could occur. If these insulin derivatives interacted with complement components, significant pathological consequences might result, considering the many events known to be mediated by the complement system (12). That such an interaction is possible was demonstrated *in vitro* by the following experiments.

*Materials.* Crystalline bovine insulin containing 24 IU/mg was obtained from Sigma (St. Louis, Mo.) and from Lilly Pharmaceutical Co. (Indianapolis, Ind.). Twice crystallized human serum albumin and highly purified gamma globulin were obtained from Mann (New York, N. Y.). Fresh guinea pig serum was absorbed twice at 0° with sheep erythrocytes to remove natural hemolytic antibody and kept at —70° until used. Sheep erythrocytes were washed in isotonic saline Veronal buffer, pH 7.4, containing 0.001 *M* $Mg^{2+}$ and 0.0015 *M* $Ca^{2+}$ and standardized to a concentration of $5\times 10^8$/ml. Rabbit antibody to boiled sheep erythrocyte stroma was used to optimally sensitize the erythrocytes for complement titrations. Rheumatoid sera, obtained from patients with rheumatoid arthritis, were tested for rheumatoid factor by standard gamma globulin coated latex agglutination tests. Glycine saline buffer used in agglutination tests contained 7.5 g/liter of glycine and 8.8 g/liter of NaCl and was adjusted to pH 8.2 with 1 *M* hydrochloric acid.

*Results.* As an initial model for study we produced aggregates of reduced insulin chains by exposing crystalline bovine insulin (20 mg/ml) to excess reduced glutathione (0.01 *M*) or to excess mercaptoethanol (2 *M*) in Veronal buffer (ionic strength 0.15, pH 8.0) for 1 hr at room temperature. These aggregates were dialyzed until all reducing agent was removed and then were exposed to guinea pig serum as a standard source of complement. An arbitrary reaction time of 1 hr at 37° was chosen. After reacting various quantities of reduced insulin aggregates with 11.8 $C'H_{50}$ units (1 $C'H_{50}$ unit is the amount of guinea pig serum that gives 50% hemolysis in our standard complement assay system), the residual complement was determined using the method described by Mayer (13). The dose–response data in Table I were obtained from these experiments. The fixation of complement was fairly rapid reaching a maximum in 15 min at 37°. The reaction was temperature sensitive. No fixation was observed after a 1-hr reaction time at 0°.

TABLE I. Complement Fixation by Reduced Insulin Aggregates.

| Aggregate concentration (μg/ml) | Offered | Fixed | Inhibition (%) |
|---|---|---|---|
| 118 | 11.8 | 1.8 | 15.2 |
| 354 | 11.8 | 4.2 | 35.6 |
| 590 | 11.8 | 8.4 | 71.2 |
| 826 | 11.8 | 10.2 | 86.4 |

Aggregates of albumin and of gamma globulin were produced by similar treatment and at a concentration of 590 μg/ml did not fix complement. Soluble, unreduced insulin was also inactive at this concentration.

The reduced insulin aggregates contained fom 0.5 to 1 reactive sulfhydryl group per 5730 g (the molecular weight of insulin). The theoretical value for complete reduction is 6. Thus, either the reduction was incomplete or some of the sulfhydryl groups were reoxidized during the long dialysis (4 days) required to remove the excess reducing agent. The latter explanation seems most likely.

Alkylation of the aggregates by treatment with iodoacetamide reduced the sulfhydryl titer to zero. However, there was no signifinant difference in the complement fixing capacity of the alkylated and nonalkylated preparations.

Treatment with 50% acetic acid solubilized most of the aggregates. Separation of the supernatant followed by neutralization of the excess acid yielded a precipitate which showed complement fixing capacity similar to that of untreated aggregates.

We also prepared aggregates by mercaptoethanol reduction in the presence of EDTA (0.005 *M*). These aggregates differed in both physical and chemical properties from aggregates prepared in the absence of EDTA.

TABLE II. Agglutination of Reduced Insulin Aggregates by Rheumatoid Serum.

| Insulin aggregate treatment | Exposed to: | 1/10 | 1/20 | 1/40 | 1/80 | 1/160 | 1/320 | Control |
|---|---|---|---|---|---|---|---|---|
| Gamma globulin | Rheumatoid serum | 4+ | 3+ | 3+ | 2+ | 0 | 0 | 0 |
| Gamma globulin, followed by 3 washes | Rheumatoid serum | 3+ | 3+ | 3+ | 2+ | ± | 0 | 0 |
| Glycine saline buffer | Rheumatoid serum | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Gamma globulin | Normal serum | ± | 0 | 0 | 0 | 0 | 0 | 0 |

They were much more finely divided and had greater complement fixing capacity. Amino acid analysis showed that the aggregates prepared in the presence of EDTA were composed of variable quantities of A and B chains. Aggregates prepared in the absence of EDTA were 70%–80% B chain. However, because of the difference in the physical state of these aggregates, the difference in complement fixing capacity cannot be correlated with the difference in chain composition.

The complement system is known to consist of nine distinct serum proteins (12). Interaction of any one or more than one of these components with the reduced insulin aggregates could account for the complement fixation data shown in Table I. The uptake by reduced insulin aggregates of a partially purified preparation of the first complement component, C'1a, made by the precipitation method of Nelson (14) was studied. This preparation is known to contain other serum proteins, but it is functionally pure for C'1a studies since it does not contain any other complement components. After incubation of insulin aggregates with this C'1a preparation, significant amounts were fixed. The C'1a that was fixed was still biologically active since C'2, a natural substrate of C'1a (15), was consumed by reduced insulin aggregates which had been exposed to C'1a but was not affected by untreated aggregates.

A possible mechanism for the fixation of C'1a by reduced insulin aggregates could be an initial adsorption of gamma globulin to produce a complex which then could fix C'1a analogously to fixation by antigen–antibody complexes (12). In order to detect gamma globulin on the surface of insoluble insulin aggregates we used a modification of the standard agglutination test for rheumatoid factor (an antibody to gamma globulin). We performed the test in the following way: Reduced insulin aggregates at a concentration of 1 mg/ml were exposed to a solution of gamma globulin (10 mg/ml) or to glycine–saline buffer for 2 hr at room temperature. The mixtures were centrifuged; supernatants were decanted; and the treated aggregates were resuspended in glycine–saline buffer at a concentration of 1 mg/ml. Serum containing rheumatoid factor was diluted and an equal volume (0.2 ml) of the treated aggregate suspensions were added and mixed. A sample of normal serum was also used. Positive agglutination observed visually after incubation for 1 hr at room temperature was quite striking, and agglutinated aggregates were relatively resistant to mechanical disruption. The control samples contained buffer but no serum and were negative.

The results shown in Table II indicate that reduced insulin aggregates can adsorb gamma globulin and that the adsorbed gamma globulin was not removed by three glycine–saline washes.

*Summary.* These experiments show that reduced insulin aggregates react with the complement system and with gamma globulin. Complement fixation may be a direct interaction between the aggregates and one or more of the complement components, but more likely it is mediated by adsorbed gamma globulin. The aggregates are soluble in 50% acetic acid, insoluble in neutral solution, and can fix complement whether or not they have titratable sulfhydryl groups. They contain both A and B chains. The relationship between complement fixing capacity of the aggregates and their physical and chemical state is under investigation, as are the *in vivo* effects of these aggregates. These *in vitro*

results support the proposed hypothesis that the renal and vascular lesions observed in diabetes are produced by a mechanism involving insulin derivatives, gamma globulin, and complement components and serve as a convenient model for further study.

1. Berns, A. W., Owens, C. T., Hirata, Y., and Blumenthal, H. T., Diabetes **11**, 308 (1962).
2. Burkholder, P. M., Diabetes **14**, 755 (1965).
3. Unanue, E. and Dixon, F. J., Advan. Immunol. **6**, 1 (1966).
4. Lachmann, P. J., Müller-Eberhard, H. J., Kunkel, H. G., Paronetto, F. J., J. Exptl. Med. **115**, 63 (1962).
5. Izzo, J. L., Bartlett, J. W., Roncone, A., Izzo, M. J., and Bale, W. F., J. Biol. Chem. **242**, 2343 (1967).
6. Westman, S., Biochem. J. **106**, 543 (1968).
7. Williams, R. H. and Einsinck, J. W., Diabetes **15**, 623 (1966).
8. Pruitt, K. M., Cantrell, J., and Boshell, B. R., Biochim. Biophys. Acta **115**, 329 (1966).
9. Meek, J. C., Doffing, K. M., and Bolinger, R. E., Diabetes **17**, 61 (1968).
10. Rasio, E. A., Hampers, C. L., Soeldner, J. S., and Cahill, G. F., Jr., J. Clin. Invest. **46**, 903 (1967).
11. Gjedde, F., Acta Physiol. Scand. **70**, 57 (1967).
12. Müller-Eberhard, H. J., Advan. Immunol. **8**, 2 (1968).
13. Mayer, M. M., *in* "Experimental Immunochemistry" (E. A. Kabat and M. M. Mayer, eds.), 2nd ed., p. 133 Thomas, Springfield, Illinois (1961).
14. Nelson, R. A., Jensen, J., Gigli, I., and Tamura, N., Immunochemistry **3**, 111 (1966).
15. Stroud, R. M., Austen, K. F., and Mayer, M. M., Immunochemistry **2**, 219 (1965).

Received Sept. 19, 1968. P.S.E.B.M., 1969, Vol. 130.

## Bactericiadl Activity of Fixed Phagocytes in Irradiated and Unirradiated Mice Treated with Enodtoxin* (33591)

HENRYKA B. JOHNSON AND CAROLYN W. HAMMOND
*Department of Microbiology, University of Illinois at the Medical Center, Chicago, Illinois 60680*

Smith *et al.* (1) reported that a single injection of bacterial endotoxin given 24 hr before X-irradiation resulted in a significant increase in survival in mice. In a subsequent study, they showed that there was an increased resistance to experimental infection and more rapid mobilization of granulocytes in peripheral blood (2) due to an earlier recovery of the cellular elements in bone marrow (3). Ainsworth and Hatch (4) confirmed these studies and also found a significantly lower incidence of endogenous infection in mice treated with endotoxin. Savage (5) showed that endotoxin did not prevent damage to bone marrow and spleen in mice but hastened their recovery. The bone marrow of endotoxin-treated mice and controls looked the same histologically for the first 3 days after X-irradiation. On the fifth day foci of neutrophils were observed but recovery was not complete until days 17–18. Without streptomycin treatment, controls were dead by day 12. Perkins *et al.* (6) found a significant increase in intracellular digestion of chicken erythrocytes by peritoneal phagocytes obtained from irradiated mice treated with endotoxin. They were unable, however, to demonstrate that endotoxin increased the survival of irradiated mice.

It had previously been established that death in animals receiving 600–800 R X-irradiation was due to leukopenia, hemorrhage, anemia and infection (7). Bacteremia in irradiated animals was reported to be due to the inability of phagocytes to destroy ingested microorganisms rather than an inability to ingest bacteria (8).

The experiments reported here were undertaken to determine whether or not the lower

* Supported by funds from the Graduate College, University of Illinois at the Medical Center.