The Effective Molecular Titration of the Early Components of Dog Complement[1] (34635)

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Although titrations of whole dog complement (1, 2) have been available, no data presently exist on the effective molecular titration of the components of dog complement with stable cellular intermediates derived from the guinea pig (3) or dog complement systems. Previous workers have demonstrated that a cellular intermediate carrying the fourth component of guinea pig complement ($C4^{gp}$) cannot be used to detect and measure the second component of human ($C2^{hu}$) (4, 5) or rabbit (6) complement, and a similar observation has now been made for the second component of dog complement ($C2^{dog}$)[2]. Fourth component of dog complement ($C4^{dog}$) cannot be measured with sequences employing second component of guinea pig or human origin. Of additional note is the finding that the measurement of $C4^{dog}$ and $C2^{dog}$ is accomplished more effectively with an intermediate formed with homologous rather than the heterologous, guinea pig first component ($C1^{gp}$). Finally, a late incompatibility, involving components acting after the first three, has been recognized between guinea pig and dog complement.

Materials and Methods. The sources and methods of handling sheep erythrocytes and guinea pig serum have been described (6). Mongrel dogs weighing 15 to 20 kg were used as sources of dog serum. The serum specimens were separated from the fibrin clot by standing at 4° for 1 hr, then were centrifuged at the same temperature, and stored in individual samples at —70°. The methods for preparing Veronal-buffered saline, pH 7.5, 0.145 *M*, containing 0.1% gelatin, 0.00015 *M* Ca^{2+} and 0.0005 Mg^{2+} (GVB^{2+}), and dextrose–Veronal-buffered saline, 0.075 *M*, with the same concentration of gelatin and cations (D-GVB^{2+}) have been reported (7). Disodium ethylenediaminetetraacetate (EDTA) buffers were prepared in GVB to a final concentration of 0.01 and 0.04 *M* EDTA (8). The methods of preparation of diethylaminoethyl (DEAE)- and carboxymethyl (CM)-cellulose, have been previously described (8, 9). Diisopropyl fluorophosphate (DFP), molecular weight 184.15, reagent grade (Aldrich Chemical Co., Inc., Milwaukee, Wis.) was handled as described by Becker (10).

Isolation of partially purified early components of dog complement. First component of dog complement ($C\bar{1}^{dog}$) was obtained by precipitating the euglobulin fraction of whole dog serum at 0°, pH 7.5, and a conductivity equivalent to 0.04 *M* NaCl by the addition of a pH 7.5, 0.005 *M* phosphate buffer (8), containing 0.0015 *M* Ca^{2+}. After the mixture was stirred gently for 3 hr at 0°, the precipitate was separated from the supernatant by centrifugation at 12,100*g* for 30 min at 0°. The precipitate was washed twice in the starting buffer and dissolved in 0.3 *M* saline in phosphate buffer, pH 7.5, with 0.0015 *M* $CaCl_2$ added. More than 95% of the $C\bar{1}$ in the starting material was recovered.

Second component of dog complement ($C2^{dog}$) was isolated from the supernatant re-

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[2] The nomenclature used conforms to that agreed upon by the World Health Organization (1968). [Bull. W. H. O. 39, 935] Sheep erythrocytes (E), sensitized with rabbit antibody (A), react with the components of complement (C) in the sequence C1, 4, 2, 3, 5, 6, 7, 8, 9. Fragments of individual components are subscripted with letters. The activated state of a component is signified by a bar above the component number, but in this paper this convention is employed only for components in the fluid phase and not for those that are cell-bound. In addition to this convention, the species of origin of a given component can be indicated by a superscript (hu, human; gp, guinea pig; dog, dog).

maining after precipitation of $C\bar{1}^{dog}$. The supernatant was adjusted to a conductivity equivalent to 0.085 *M* NaCl, 0.001 *M* disodium ethylenediaminetetraacetate (EDTA) was added and the material was concentrated by pressure filtration to the original volume of serum (10 ml). Diisopropyl fluorophosphate was then added to the supernatant to yield a final concentration of $5 \times 10^{-3} M$, and following incubation at 37° for 60 min, the material was applied to a DEAE column (2.5 × 25 cm) equilibrated to the same conditions of pH and ionic strength as the starting material. The column was washed with the starting buffer and then eluted with a linear increase in NaCl from 0.085 to 0.3 *M* in the same buffer. The $C2^{dog}$ was present in the initial effluent. This effluent was pooled and concentrated by pressure filtration to the original volume of serum and reapplied to another DEAE-cellulose column equilibrated at the same pH and conductivity as the initial column. $C2^{dog}$ was again present in the initial effluent. After concentration the $C2^{dog}$ preparation yielded activities of approximately 1×10^{10} to 3×10^{10} effective molecules/ml with a C4 contamination of less than 1×10^{9} effective molecules/ml. The recovery of C2 was extremely poor when larger DEAE-cellulose columns were employed or when CM-cellulose chromatography was utilized.

Fourth component of dog complement ($C4^{dog}$) and inhibitor of C1 ($C\bar{1}INH^{dog}$) were eluted from the previously described DEAE column at a conductivity equivalent to 0.12 to 0.15 *M* NaCl. These fractions were pooled and concentrated by pressure filtration to 2–3 times the initial volume of serum, brought to a pH of 5.5 with 1 *N* acetic acid and diluted to a conductivity equivalent to 0.055 *M* NaCl with 0.05 *M* Na acetate buffer containing 0.001 *M* EDTA and applied to a 2.5 × 25 cm CM-cellulose column. The $C\bar{1}INH^{dog}$ passed directly through the CM column (8) and the $C4^{dog}$ was subsequently eluted at a conductivity equivalent to 0.12 to 0.15 *M* NaCl. The $C4^{dog}$ preparation was then pooled and concentrated by pressure filtration. Such preparations yielded $C4^{dog}$ activity between 2×10^{10} and 4×10^{10} effective molecules/ml; they were free of detectable C2 by hemolytic titration and of C3 by immune adherence (4, 12).

Cellular intermediates of the hemolytic system and titration of complement components. Sheep erythrocytes were sensitized (9) with commercial rabbit antibody to sheep red cells. $EAC1^{dog}$ or $EAC1^{gp}$ cells were prepared by mixing EA at a concentration of 5×10^{8} in D-GVB^{2+} with an equal volume of partially purified $C\bar{1}^{dog}$ or $C\bar{1}^{gp}$ diluted to provide 200 effective molecules/cell; after incubation at 30° for 45 min, the cellular intermediates were washed three times in D-GVB^{2+} and stored in the same buffer with 100 μg of penicillin and streptomycin (9). The $EAC14^{dog}$ cells were prepared from either $EAC1^{dog}$ or $EAC1^{gp}$ by the method described by Borsos and Rapp (13), utilizing dog complement diluted 1:5 with 0.01 *M* EDTA; or by incubating $EAC1^{dog}$ with partially purified $C4^{dog}$ diluted in D-GVB^{2+} at 30° for 30 min. $EAC4^{dog}$ cells were obtained by incubating $EAC14^{dog}$ in 0.01 *M* EDTA-GVB according to the method of Borsos and Cooper (14). $EAC4^{hu}$ cells were prepared from $EAC1^{gp}4^{hu}$ by the same method (14). All cellular intermediates were washed and stored as described for $EAC1^{dog}$. The procedures for the effective molecular titrations of C1 (15), C4 (9), C2 (13), $C\bar{1}INH$ (11) and for $C\bar{1}$ transfer (16) were performed as previously described.

Results. Whole complement levels in the serum of 5 mongrel dogs, assessed by the standard 7.5-ml hemolytic assay (3), were 16, 23, 31, 39, and 42 CH_{50} units/ml.

Titration of $C1^{dog}$ and $C\bar{1}INH^{dog}$. The titration of $C1^{dog}$ in whole serum, using $EAC4^{dog}$ cells made from either $EAC1^{dog}$ or $EAC1^{gp}$, is illustrated in Fig. 1A and B. Approximately 30 effective molecules of $C2^{dog}$ were employed to bring the $EAC14^{dog}$ cells to the $EAC142^{dog}$ stage, and they were then lysed by the addition of whole dog serum diluted 1:10 in 0.04 *M* EDTA. The titration of C1 in whole dog serum always passed to the right of the origin, while the titration of partially purified $C\bar{1}$ proceeded to the origin (Fig. 1A, B). These observations were consistently obtained with other dog sera and their partially

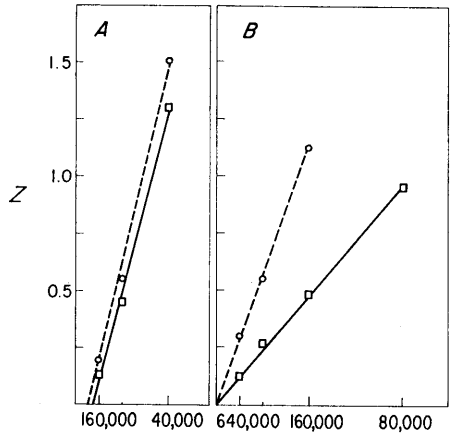

FIG. 1A. Titration of C1 in dog serum with EAC4^{dog} cells derived from EAC1^{gp} (○) and EAC1^{dog} (□). *Z* refers to the proportional number of hemolytically active sites formed per erythrocyte (3). (B) Titration of partially purified C1^{dog} with the same two populations of EAC4^{dog} cells.

purified $C\bar{1}$.

The inhibitor of $C\bar{1}^{dog}$ was measured functionally in both the pseudoglobulin supernatant and following purification by DEAE- and CM-cellulose chromatography. The titration of $C\bar{1}INH^{dog}$ (11) was based on its ability to inhibit fluid phase $C\bar{1}^{gp}$ as detected with EAC4^{hu} (Fig. 2).

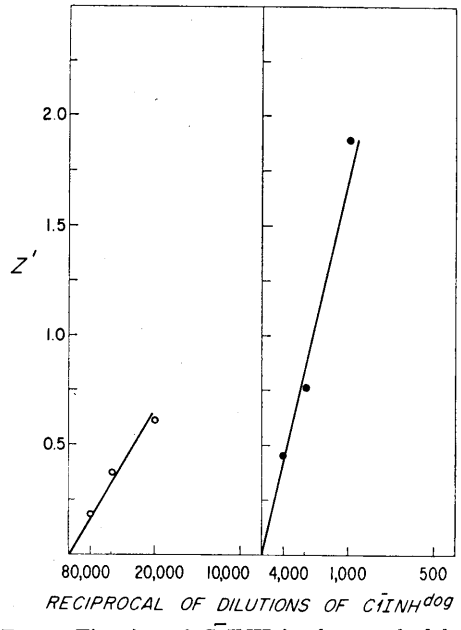

FIG. 2. Titration of $C\bar{1}INH$ in the pseudoglobulin fraction of dog serum (left) and in its partially purified form (right). Z′ refers to the average number of inhibited sites per erythrocyte (11).

Titration of C4^{dog}. The hemolytic titration of C4 in whole dog serum with either EAC1^{dog} or EAC1^{gp} intermediates is depicted in Fig. 3A, B. The EAC14 cells formed were interacted with approximately 10 effective molecules of C2^{dog} and the reaction was brought to completion by the addition of dog serum diluted 1:10 in 0.04 *M* EDTA. Because of C4 contamination, the number of effective molecules of C2 employed was limited so that the reagent lysis was less than 10%. Compared to EAC1^{gp}, EAC1^{dog} cells gave a 10-fold higher C4^{dog} titer. Similar differences in titer were observed when partially purified C4^{dog} was assayed with the same two cell populations.

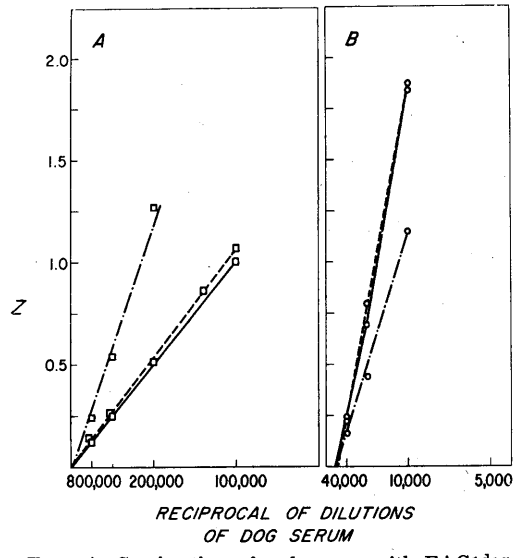

FIG. 3A. C4 titration of 3 dog sera with EAC1^{dog}. (B) C4 titration of 3 dog sera with EAC1^{gp}.

Titration of C2^{dog}. Effects of varying the species of $C\bar{1}$ employed in the preparation of EAC4 cells from which EAC14^{dog} intermediate was derived. Two populations of EAC1 cells, EAC1^{dog} and EAC1^{gp}, were used to prepare EAC4^{dog} cells by the method described by Borsos and Rapp (13); both cell populations were converted to EAC1^{dog}4^{dog} by

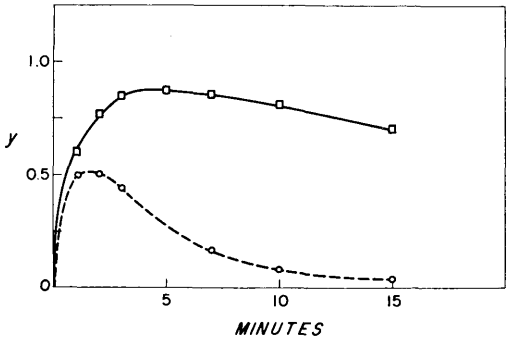

FIG. 4. Kinetics of EAC142 formation of EAC14^{dog} cells derived from EAC1^{dog} (□) and EAC1^{gp} (○). y refers to the percentage lysis with 1 = 100% (3).

the addition of 200 effective molecules/cell of partially purified $\overline{C1}^{dog}$. The number of $\overline{C1}$ molecules present in the EAC1^{gp} or EAC1^{dog} and in the EAC14^{dog} derived from each of them was determined by $\overline{C1}$ transfer to EAC4^{hu} recipient cells. The original EAC1^{gp} and EAC1^{dog} cells contained 66 C1 sites (SAC1)/cell, while the EAC14^{dog} cells derived contained 37 and 34 SAC1, respectively. Following conversion to the EAC142 stage by the addition of C2^{dog}, the cells were sampled at specific time intervals and lysed by the addition of dog serum diluted 1:15 in 0.04 *M* EDTA. As depicted in Fig. 4, the two cell populations gave maximum lysis (c_{max}) at different time intervals (t_{max}). When used at the appropriate t_{max} time for each cell population, the C2 titer of either whole dog serum or partially purified C2 was greater with the EAC14^{dog} intermediate developed from EAC1^{dog} (Fig. 5A, B). Guinea pig C2 at 25 effective molecules/cell was incapable of preparing the EAC14^{dog} cells for lysis by either guinea pig or dog serum diluted in 0.04 *M* EDTA.

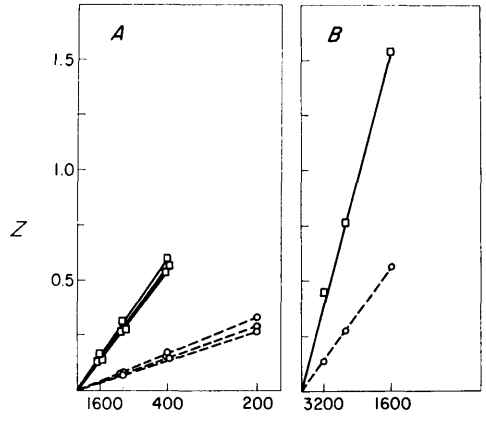

FIG. 5A. Titration of C2 in 3 dog sera employing EAC14^{dog} cells derived from either EAC1^{dog} (□) or EAC1^{gp}. (○). (B) Titration of C2 in its partially purified form employing EAC14^{dog} cells derived from either EAC1^{dog} (□) or EAC1^{gp} (○).

Effects of varying the source of C-EDTA on the lysis of EAC142^{dog} cells. EAC14^{dog} cells prepared from EAC1^{dog} were incubated with approximately 3 effective molecules of C2^{dog}/cell. At specific time intervals, aliquots of the reaction mixture were sampled and added to a source of C-EDTA derived from either guinea pig or dog serum diluted in 0.04 *M* EDTA. As illustrated in Fig. 6A, C-EDTA derived from dog serum was able to lyse the EAC142^{dog} cells; whereas, the same cells were resistent to lysis by guinea pig C-EDTA. In order to examine further the differential activity of dog and guinea pig C-

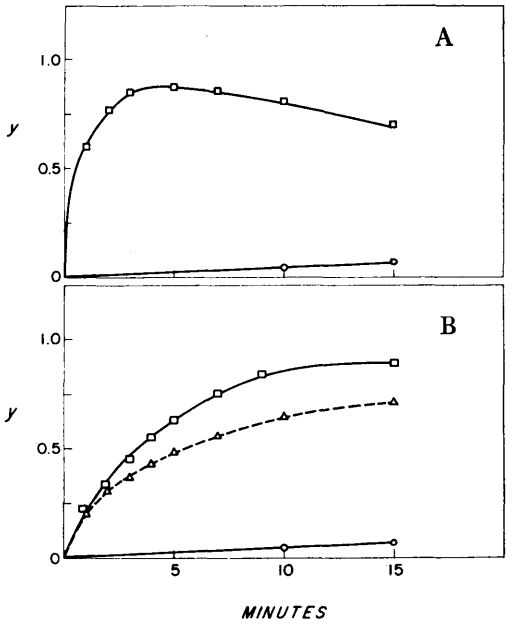

FIG. 6A. Kinetics of EAC142^{dog} formation and subsequent lysis by dog (□) and guinea pig C-EDTA (○). (B) Lysis of EAC142^{dog} prepared from partially purified components by dog (□), human (△), and guinea pig (○) C-EDTA.

TABLE I. Titer of the Early Components of Dog Complement.

Dog serum	CH_{50} (units/ml)	C1 (units/ml)	C4 (units/ml)	C2 (units/ml)
III	41.6	88,000	120,000	250
IV	23	100,000	150,000	—
V	39	100,000	228,000	160
VI	16	123,000	70,000	200
VII	31	75,000	100,000	—

EDTA, $EAC14^{dog}$ cells were prepared from partially purified components. $EAC1^{dog}$ cells, made with 25 effective molecules of partially purified $\overline{C1}$, were converted to the EAC14 stage by the addition of 75 effective molecules/cell of partially purified $C4^{dog}$. Following incubation for specific intervals of time, with a limited concentration of partially purified $C2^{dog}$, aliquots of the cells were taken and added to a source of C-EDTA derived from dog, human, or guinea pig serum diluted 1:15, 1:15, and 1:22, respectively, in 0.04 *M* EDTA. Dog and human C-EDTA proved capable of lysing the $EAC142^{dog}$ cells, whereas guinea pig C-EDTA was unable to do so (Fig. 6B).

Discussion. Although titrations of whole dog complement (1, 2) have been published, observations on the effective molecular titration of the early components of dog complement (Table I) have not previously been available. A striking characteristic of the titrations conducted so far is the efficiency of the homologous system in contrast to that which employs a guinea pig component or components at certain points in the hemolytic sequence. This characteristic was demonstrated when $\overline{C1}^{gp}$ was employed to make an $EAC1^{gp}$ cell for the titration of $C4^{dog}$; when the same cell was used in the initial step in the production of $EAC14^{dog}$ for the titration of $C2^{dog}$; and when guinea pig C-EDTA was utilized to complete the lysis of $EAC142^{dog}$ cells.

The greater effectiveness of $EAC1^{dog}$ cells as contrasted to those made from $\overline{C1}^{gp}$ in titrating $C4^{dog}$ is illustrated in Fig. 3. The two populations of EAC1 cells were prepared with identical inputs of $\overline{C1}$, from a single pool of EA. The $\overline{C1}$ assays, from which the $\overline{C1}$ inputs were calculated, were performed under the optimal conditions determined for each species. $\overline{C1}^{gp}$ was measured with $EAC4^{hu}$, $C2^{gp}$, and guinea pig C-EDTA (11), while $\overline{C1}^{dog}$ was titered with $EAC4^{dog}$ derived from $EAC1^{gp}$, since this intermediate yielded the higher value (Fig. 1). Transfer experiments to $EAC4^{hu}$ to determine the number of $\overline{C1}$ sites formed revealed identical numbers for the $EAC1^{gp}$ and $EAC1^{dog}$.

An unexpected finding was that the species of $\overline{C1}$, guinea pig or dog, on the EAC1 intermediate employed to produce $EAC4^{dog}$ cells by the method of Borsos and Rapp (13) influenced the activity of these cells. In this method, EAC1 cells are incubated with C-EDTA with the resultant fixation of C4 and loss of $\overline{C1}$ so as to yield the EAC4 intermediate. Conversion of $EAC4^{dog}$ cells to $EAC14^{dog}$ by the addition of $\overline{C1}^{dog}$ produced cellular intermediates displaying different t_{max} values (Fig. 4), and yielding significantly different $C2^{dog}$ titrations depending on the species of $\overline{C1}$ on the parent EAC1 cell (Fig. 5). When the original cell was prepared with $\overline{C1}^{dog}$, a higher C2 titer was obtained with the derived $EAC14^{dog}$ cells than when the original cell was $EAC1^{gp}$. This was true for the measurement of C2 in either whole dog serum or after its partial purification by DEAE-cellulose chromatography. These findings suggest either that the cell-bound homologous $\overline{C1}$ placed more $C4^{dog}$ onto the cell, that the C4 was positioned in such a way so as to create a more effective site on the subsequent addition of $\overline{C1}^{dog}$, or that residual $\overline{C1}^{gp}$ or its subunits were present and interfered with the effective introduction of $\overline{C1}^{dog}$ at the appropriate $SAC4^{dog}$ site. That the number of $C1^{dog}$

sites present on the two populations of EAC14 cells was the same was indicated by transfer analysis. That the number of EAC14 sites were, at least, comparable in the two cell populations is suggested by Fig. 4 in which the t_{max} of $EAC14^{dog}$ cells derived from $EAC1^{gp}$ was actually shorter than that of the more effective $EAC14^{dog}$ cells derived from $EAC1^{dog}$. Irrespective of the mechanism, the higher titer of $C4^{dog}$ obtained with $EAC1^{dog}$, and of $C2^{dog}$ achieved with $EAC14^{dog}$ cells derived from $EAC1^{dog}$ indicate the importance of using the homologous system. These observations are reminiscent of the recent findings of Gigli and Austen (17, 18) that the fluid-phase inactivation of guinea pig or human C4 or C2 is most effectively accomplished with homologous $\overline{C1}$.

A unilateral incompatibility between $C2^{hu}$ and $C4^{gp}$ (4, 5) and a bilateral incompatibility between C4 and C2 guinea pig and rabbit (6) has previously been described. A similar incompatibility was observed to exist between $C4^{dog}$ and $C2^{gp}$. $C2^{gp}$ was unable to convert $EAC14^{dog}$ cells to an EAC142 intermediate which was susceptible to lysis by addition of either guinea pig or dog C-EDTA.

The failure of C-EDTA prepared from guinea pig serum to lyse $EAC142^{dog}$ cells introduces a new species incompatibility (Fig. 6A, B). As assessed by immune adherence, the use of $C3^{gp}$ to convert some $EAC142^{dog}$ cells to the $EAC142^{dog}3^{gp}$ state failed to render the cells susceptible to lysis by guinea pig C-EDTA, whereas dog serum at its usual dilution in 0.04 *M* EDTA retained its capacity to lyse the cells. This incompatibility may reside between $C2^{dog}$ and the ill-defined role of C2 in the fixation of the fifth component of complement (19) or alternatively in the inability of $C3^{dog}$ convertase to activate $C3^{gp}$ for its role in the hemolytic sequence.

Summary. Effective molecular titrations of C1, C4, and C2 revealed values ranging from 75,000 to 123,000 for C1, from 70,000 to 228,000 for C4, and from 160 to 250 for C2 in the serum of five mongrel dogs. A striking feature of these experiments has been the efficiency of the homologous system as compared to that which employs guinea pig components. Complete or partial functional incompatibilities were demonstrated when: (i) $\overline{C1}^{gp}$ was employed to make EAC1 intermediate used for the titration of $C4^{dog}$; (ii) when $EAC1^{gp}$ was used in the formation of the $EAC4^{dog}$ cells which were converted to $EAC14^{dog}$ cells for the titration of $C2^{dog}$; (iii) when $C2^{gp}$ was introduced to prepare $EAC14^{dog}$ cells for lysis by C-EDTA, and (iv) when lysis of $EAC142^{dog}$ cells was attempted with C^{gp}-EDTA.

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1. Favour, C. B., Murray, J. E., Wemyss, C. T., Jr., Colodny, A., and Miller, B. F., Proc. Soc. Exp. Biol. Med. **83**, 352 (1953).

2. Simonsen, M., Acta Pathol. Biol. Scand. **32**, 36 (1953).

3. Kabat, E. A., and Mayer, M. M., "Experimental Immunochemistry," 1st ed. Thomas, Springfield, Ill. (1948).

4. Nelson, R. A., *in* "The Inflammatory Process" (B. W. Zweifach, L. Grant, and R. T. McCluskey, eds.), p. 819. Academic Press, New York (1965).

5. Austen, K. F., and Russell, P. S., Ann. N. Y. Acad. Sci. **129**, 657 (1966).

6. Kempf, R. A., Gigli, I., and Austen, K. F., J. Immunol. **102**, 795 (1969).

7. Borsos, T., and Rapp, H. J., J. Immunol. **91**, 826 (1963).

8. Nelson, R. A., Jensen, J., Gigli, I., and Tamura, N., Immunochemistry **3**, 111 (1966).

9. Ruddy, S., and Austen, K. F., J. Immunol. **99**, 1162 (1967).

10. Becker, E. L., J. Immunol. **84**, 299 (1960).

11. Gigli, I., Ruddy, S., and Austen, K. F., J. Immunol. **100**, 1154 (1968).

12. Gigli, I., and Nelson, R. A., Jr., Exp. Cell Res. **51**, 45 (1968).

13. Borsos, T., and Rapp, H. J., J. Immunol. **99**, 263 (1967).

14. Borsos, T., and Cooper, M. C., Proc. Soc. Exp. Biol. Med. **107**, 277 (1961).

15. Borsos, T., Rapp, H. J., and Walz, U. L., J. Immunol. **92**, 108 (1964).

16. Borsos, T., and Rapp, H. J., J. Immunol. **95**, 559 (1965).

17. Gigli, I., and Austen, K. F., J. Exp. Med. **129**, 679 (1969).

18. Gigli, I., and Austen, K. F., J. Exp. Med. **130**, 833 (1969).

19. Cooper, M. R., Fed. Proc., Fed. Amer. Soc. Exp. Biol. **28**, 818 (1969).

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