INTERACTION OF β1H GLOBULIN WITH CELL-BOUND C3b: Quantitative Analysis of Binding and Influence of Alternative Pathway Components on Binding*

By DANIEL H. CONRAD‡, JAIME R. CARLO‡, AND SHAUN RUDDY

(From the Division of Immunology and Connective Tissue Diseases, Medical College of Virginia, Health Sciences Division, Virginia Commonwealth University, Richmond, Virginia 23298)

Participation of C3b, the major cleavage product of C3, in both the classical and alternative pathways of complement activation is modulated by several control proteins (1–3). Two of these, C3b inactivator (C3bINA)[1] (4, 5) and β1H-globulin (β1H) (2), have been extensively purified and characterized. It is now apparent that C3bINA is a protease, and that it blocks the biologic activities of C3b by cleaving peptide bonds in this molecule (1, 4, 6). The second protein, β1H, potentiates the activity of C3bINA; indeed, recent evidence indicates an absolute requirement for β1H in the cleavage of fluid phase C3b by C3bINA (4). In addition, highly purified β1H by itself both directly inhibits the activity of C3b (4) and accelerates the rate of decay of the alternative pathway convertases, $\overline{C3bB}$ and $\overline{C3bBP}$ (2, 7).

Of great interest is the mechanism by which β1H exerts these effects. No proteolytic activity that can be directly ascribed to β1H has been found. Direct binding of β1H to C3b and subsequent steric interference with the interaction of C3b with factor B and/or C5 is the most straightforward explanation; two lines of evidence, fluid phase depletion and agglutination by antibody to β1H of EAC43 previously exposed to β1H (8), had indicated that such binding occurs. More recently, both this laboratory (9) and another (10) have presented further information about the binding of β1H to C3b-coated particles. The studies reported here give quantitative measurement of strength and valence of this binding, examine the influence of fluid phase C3 and C3b on it, and determine the effects that factor B (B) and properdin (P), which also bind to C3b, have on the binding of β1H to C3b-coated cells.

Materials and Methods

Reagents. Bio-Rad Ag-1-X-10 (chloride form), Bio-Rex 70, electrophoresis grade polyacrylamide, bis-acrylamide, and sodium dodecyl sulfate (SDS) were obtained from Bio-Rad Laborato-

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[1] *Abbreviations used in this paper:* A, rabbit antibody; B, factor B; β1H, β1H globulin; C3bINA, C3b inactivator; $DGVB^{++}$, equal volumes of GVB^{++} and $D5W^{++}$; $D5W^{++}$, 5% dextrose in water; E, sheep erythrocytes; FITC, fluorescein isothiocyanate; GVB^{++}, 0.1% gelatin veronal buffer; P, properdin; SBTI, soybean trypsin inhibitor; SDS, sodium dodecyl sulfate; VBS, veronal-buffered saline.

ries, Richmond, Calif. Radioiodide, both ^{131}I and carrier-free ^{125}I, was obtained from Amersham Corp., Arlington Heights, Ill. Trypsin and soybean trypsin inhibitor (SBTI) were obtained from Worthington Biochemical Corp., Freehold, N. J. Bovine serum albumin (Cohn Fraction V) was obtained from Calbiochem, San Diego, Calif. Las-R human complement C3 reagent kit was purchased from Hyland Diagnostics Div., Travenol Laboratories, Costa Mesa, Calif., and Sepharose 4B from Pharmacia Fine Chemicals, Inc., Piscataway, N. J.

Buffers. Isotonic veronal-buffered saline (VBS) containing 0.00015 M Ca^{++}, 0.0005 M Mg^{++}, and 0.1% gelatin (GVB^{++}) and 5% dextrose in water containing the same concentrations of divalent cations ($D5W^{++}$) were mixed in equal volumes; the resulting buffer, ionic strength of 0.065 and pH 7.4, is referred to as $DGVB^{++}$ and was used in all binding studies. GVB^{--} was made as described above except that the divalent cations were not present. A stock solution of 0.086 M EDTA, pH 7.5, was diluted in GVB^{--} to prepare 0.04 M EDTA GVB^{--}.

Component Purification. Guinea pig C1 (11) and human C2 (12) were prepared as published elsewhere. Partially purified factor B was obtained by a modification of the procedure of Götze and Müller-Eberhard (13). Normal human serum was adjusted to 40% Na_2SO_4 and the resulting precipitate was redissolved and subjected to chromatography on Bio-Rex 70 (13). Properdin was prepared by a modification in the procedure of Pensky et al. (14). The high salt eluate from zymosan was dialyzed against low ionic strength buffer, and the resulting P-containing precipitate was redissolved in VBS and subjected to chromatography on a Sephadex G-200 column. B (15) and P (16) were measured hemolytically as described elsewhere. Highly purified human C3 was prepared as described by Tack and Prahl (17), and β1H was prepared as described by Whaley and Ruddy (2). C3b was prepared from the purified C3 with trypsin and SBTI as described by Bokisch et al. (18).

Antisera. Antisera to C3, B, P, and β1H were induced in goats and were subsequently used in radial immunodiffusion (19) to determine the concentration of the various components. Pooled human serum, which had been previously calibrated against purified C3, B, P, and β1H, served as the standard. In radiolabeled preparations, concentrations of C3 and β1H were determined nephelometrically with a Hyland Laser Nephelometer PDQ Instrument (Hyland Diagnostics Div.) and a Hyland LAS-R human complement C3 kit for C3 determinations. When β1H was measured nephelometrically, doubling dilutions of pooled human serum (1:12.5 to 1:400) were used as standards. The 1:12.5 dilution was first filtered through a 0.4-μm Nucleopore filter (Nucleopore Corp., Pleasanton, Calif.) before further diluting. Rabbit anti-β1H was diluted with saline and subsequently an equal volume of phosphate-buffered saline (0.01 M PO_4^{--}, 0.15 M NaCl, pH 7.4) containing 4% polyethylene glycol was added to give a final antibody dilution of 1:60. 50 μl of standards or appropriately diluted unknowns were added to 1 ml antibody dilutions and after 1 h at room temperature were examined for light scatter in the nephelometer; 50 μl of the same samples added to 1 ml of saline served as blank controls.

The purity of the ^{125}I-labeled β1H (^{125}I-β1H) (see below) was also estimated by testing the ability of the preparation to be insolubilized with the monospecific goat anti-β1H. ^{125}I-β1H was mixed with an excess of goat anti-β1H, and after 30 min at 37°C the β1H-anti-β1H complexes were precipitated by adding a predetermined optimal amount of rabbit anti-goat IgG (Atlantic Antibodies, Westbrook, Maine). After 1 h at 37°C and overnight at 4°C, the complexes were washed three times with saline and the radioactivity remaining with the precipitate was measured. All β1H preparations tested in this manner were 80–87% precipitable by the anti-β1H.

Immunofluorescent Staining. The globulin fraction of goat anti-β1H was conjugated with fluorescein isothiocyanate (FITC) according to the method of Herbert et al. (20). The fluorescein to protein ratio (molar) of the final preparation was 2:1. Approximately 100 μg of β1H was covalently linked to Sepharose 4B (see below), and subsequently the specificity of the anti-β1H was confirmed in blocking experiments whereby fluorescent staining of the Sepharose-bound β1H was inhibited by reacting the FITC-anti-β1H with highly purified β1H.

The cells were examined for fluorescence by using a Zeiss photomicroscope II (Carl Zeiss, Inc., New York) with a HBO 200 light source, FITC excitation primary filter, and a 530-nm secondary filter.

Radioiodination. Highly purified β1H and C3 were radioiodinated with ^{125}I (carrier free) or ^{131}I by the use of the chloramine T procedure (21). Unbound iodide was removed by ion exchange chromatography with Bio-Rad Ag 1-X-10 (chloride form) and overnight dialysis versus VBS. In the final preparations the radioiodide was 90–98% precipitable with 10% trichloroacetic acid. The specific activities obtained were in the range of 2–5 $\times$ 10^6 cpm/μg and 0.1–1.0 $\times$ 10^6 cpm/μg for

β1H and C3, respectively. Bovine serum albumin (Cohn Fraction V) was added to the stock solutions of the radiolabeled proteins, and storage was at −70°C. The correspondences between ^{125}I and ^{131}I counts and numbers of molecules of β1H or C3 were calculated from the specific activities of the iodinated proteins, using Avogadro's number and mol wt of 185,000 and 150,000 daltons (2) for C3 and β1H, respectively.

Cellular Intermediates. Sheep erythrocytes (E) were sensitized with rabbit antibody (A), and EAC4 were prepared and stored at −70°C in a glycerol-containing medium as described elsewhere (22). EAC4 were thawed as needed. EAC14 and $EAC14^{ox}2$ were prepared by using guinea pig C1 and human C2 which had been oxidized with I_2 (23). EAC43 were prepared with nonoxidized C2 as described previously (15); the EAC43 did not lyse when exposed to a C3-9 source (rat serum diluted 1:15 in 0.04 M EDTA GVB^-). $EAC14^{ox}23$ and $EAC14^{ox}2$ ^{131}I-C3 were prepared by incubating $EAC14^{ox}2$ in $DGVB^{++}$ with purified C3 (either unlabeled or ^{131}I-labeled) for 30 min at 37°C followed by three washes with $DGVB^{++}$.

Counting Technique. Measurements of ^{125}I, ^{131}I, and ^{22}Na were made in a dual channel gamma counter (model 1185, Searle Analytic, Chicago, Ill.). When all three isotopes were used simultaneously, the samples were counted twice, first for ^{125}I and ^{22}Na and then for ^{131}I and ^{22}Na. Channel settings were adjusted such that 0.1% or less spillover of the lower energy isotope (order of energy: $^{22}Na > ^{131}I > ^{125}I$) into the higher one(s) occurred. The ^{125}I cpm was thus corrected for spillover of ^{22}Na and, when necessary, ^{131}I; ^{131}I cpm was corrected for ^{22}Na spillover. Background corrections were also made for all channels.

Radioactive Binding Assays. To avoid extensive manipulation of cells, ^{22}Na was used as a volume marker for unbound ^{125}I-β1H remaining with the cells. In a typical experiment, prepared cellular intermediates were incubated with ^{125}I-β1H for 15 min at 30°C in a vol of 1 ml. The cells were then sedimented by centrifugation and 0.1 ml of supernate removed. Approximately 90% of the remaining supernate was then removed by aspiration and the cells quantitatively transferred with $DGVB^{++}$ into a clean tube. Radioactive determinations were then made on the supernatant aliquot and the cell pellet. Based on the assumption that the ratio of free ^{125}I-β1H and ^{22}Na in the incubation solution was constant, the amount of β1H bound to the cells was determined by the following formula:

$$\text{cpm } ^{125}I = \beta1H \text{ bound} = A - (x/y)(z)$$

where A is the total ^{125}I cpm in the cell pellet, x is the ^{22}Na cpm in the cell pellet, y is the ^{22}Na cpm in the supernate, and z is the ^{125}I cpm in the supernate. This method of calculation is similar to that described by Tsay and Schlamowitz (24). In situations where the ^{131}I-C3 was used to quantitate the amount of cell bound C3b, supernatant corrections with ^{22}Na were unnecessary since there was essentially no ^{131}I-C3 in the fluid phase.

In binding assays in which the objective was determination of binding parameters, $EAC14^{ox}23$ or $EAC14^{ox}2$ ^{131}I-C3 were incubated for 15 min at 30°C with various amounts (0.1–2 μg) of ^{125}I-β1H in a total vol of 1 ml, and subsequently the bound ^{125}I-β1H was determined as described above. The experimental data were then plotted according to the method of Scatchard (25).

$$r/c = nK - rK$$

where r represents the number of β1H molecules bound per cell (or alternatively per ^{131}I-C3b molecule), K is the average association constant, n the total number of binding sites per cell (or per C3b), and c is the concentration of free bindable ^{125}I-β1H. c was calculated as follows:

$$c = (\beta1H_T)(MB) - \beta1H_b,$$

where $\beta1H_T$ is total β1H added (molecules/milliliter), MB is the maximal binding ability of the β1H preparation, and $\beta1H_b$ is the number of molecules of β1H bound to the cell. In the final Scatchard plots, the best straight line for the experimental points was computed by the method of least squares using a Wang WCS-20 computer. K was obtained from the slope of this line and was converted to the more familiar liters per mole units by using Avogadro's number and a mol wt of 150,000 daltons (2) for β1H. The maximal number of β1H binding sites (n) was obtained from the intercept of the line with the abscissa. This intercept represents an infinite free concentration of β1H.

Other Analytical Procedures. Immunoelectrophoresis was performed by standard technique (26). SDS-gel electrophoresis with 7.5% separation gels was performed by the method of Laemmli

(27). Immunoadsorbents and β1H-Sepharose were prepared by linking the respective protein to Sepharose 4B by the cyanogen bromide procedure (28).

Results

Congruent Immunofluorescent Staining and Agglutination by Anti-β1H. Direct visual demonstration of both binding of β1H to $EAC14^{oxy}23$ and agglutination of such cells by anti-β1H was obtained. For this experiment, $EAC14^{oxy}23$ were prepared as described in Materials and Methods. One-half of the cell preparation (1.0×10^8 cells/ml) was then incubated for 15 min at 30°C with 12 μg β1H in a vol of 0.6 ml, and the other half with $DGVB^{++}$ alone. After washing three times with $DGVB^{++}$, the two cell populations were remixed and subsequently allowed to interact for 30 min at 25°C with fluoresceinated anti-β1H. After washing in $DGVB^{++}$, the cell mixture was mounted on glass slides and examined by both phase and fluorescent microscopy. There was gross aggregation of some, but not all, of the cells. When examined by fluorescence microscopy, only the cells that stained for β1H by immunofluorescence were agglutinated, thus indicating that the agglutination was associated with the presence of bound β1H on the cells.

Relation between β1H Binding and Amount of Surface-Bound C3b. Using ^{125}I-β1H, the dependence of β1H binding on the presence of C3b was directly examined. Populations of cells bearing varying amounts of C3b on their surfaces were made by treating $EAC14^{oxy}2$ (5×10^7 per ml) with C3 concentrations ranging from 0 to 45.6 μg/ml for 30 min at 37°C. The cells were washed three times with $DGVB^{++}$, exposed to 0.9 μg/ml of ^{125}I-β1H for 15 min at 30°C, and then washed three more times with $DGVB^{++}$. After transferring to clean tubes, the amount of ^{125}I-β1H bound was determined. $EAC14^{oxy}23$ generated with increasing amounts of C3 bound increasing amounts of ^{125}I-β1H (Fig. 1). Approximately 29,000 molecules of ^{125}I-β1H were bound per cell at the highest input of C3; this represented 37% of the available ^{125}I-β1H.

Maximum Binding Ability of ^{125}I-β1H Preparations. The proportion of ^{125}I-β1H in a given preparation which was capable of binding to C3b was examined in the following experiment using an excess of cells relative to the concentration of ^{125}I-β1H. Varying numbers of EA, EAC14, $EAC14^{oxy}2$, or $EAC14^{oxy}23$ were incubated with 0.1 μg of ^{125}I-β1H for 15 min at 30°C, and the amount of binding determined by the ^{22}Na procedure described in Materials and Methods. This procedure was used to allow the detection of low affinity binding of the ^{125}I-β1H by preventing any loss of bound ^{125}I-β1H due to washing. Significant binding of the ^{125}I-β1H occurred only with $EAC14^{oxy}23$ intermediate (Fig. 2). Even with maximal numbers of these cells, however, only 48% of the ^{125}I-β1H was bound. In similar experiments with three other β1H preparations, maximal binding abilities ranged from 30 to 62%. Addition of fresh $EAC14^{oxy}23$ to supernates containing ^{125}I-β1H which had been previously exposed to $EAC14^{oxy}23$ did not result in any additional binding of the ^{125}I-β1H, indicating that the unbound material did not have the capacity to bind to cell-bound C3b. As indicated in Materials and Methods, 80–87% of the labeled β1H preparation was precipitated by monospecific goat anti-β1H. Kinetic experiments (data not shown) indicated that binding equilibrium was reached as early as 5 min at 30°C; thus, it is clear that some of the ^{125}I-β1H was not bindable. Possible reasons for this are

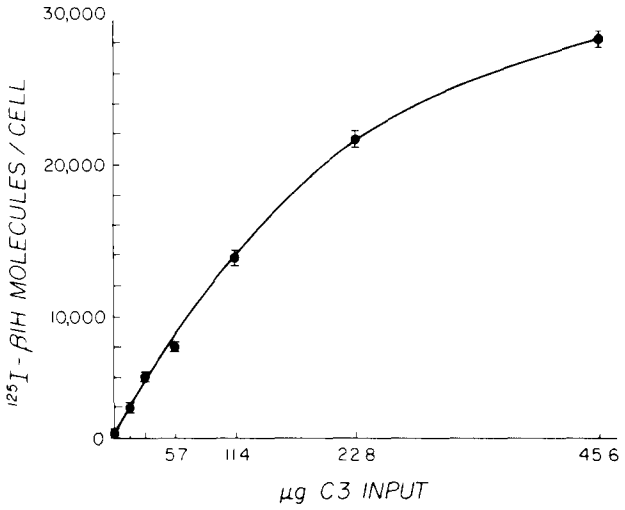

FIG. 1. ^{125}I-β1H binding by EAC14^{oxy}23 generated with increasing concentrations of C3. The bars indicate the standard error of the mean for triplicate determinations.

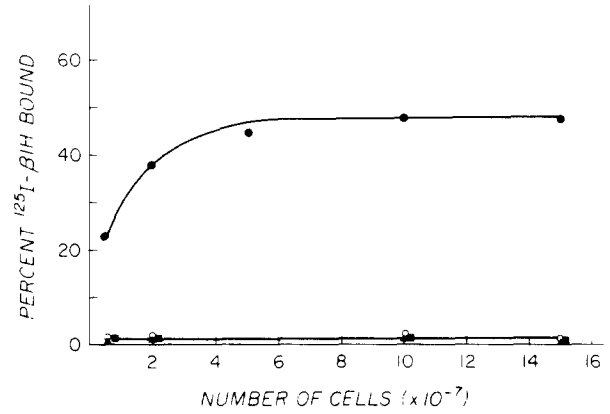

FIG. 2. Percent of ^{125}I-β1H bound by varying numbers of EA (▲), EAC14 (○), EAC14^{oxy}2 (■) or EAC14^{oxy}23 (●).

considered in the Discussion, but it should be noted here that in all of the quantitative analyses of ^{125}I-β1H binding, the maximum binding ability for the particular preparation was used in the calculations.

Quantitative Analysis of β1H Binding. When a constant number of EAC14^{oxy}23 (5×10^6 cells) is incubated for 15 min at 30°C with increasing concentrations of ^{125}I-β1H, and β1H binding initially increases rapidly and then tends to level off as the binding sites become saturated. A plot of the raw data from an experiment of this type is shown in Fig. 3a. Shown also is the same data corrected for the small amount of binding when EAC14^{oxy}2 was used instead of the EAC14^{oxy}23.

To estimate the binding constants of this reaction, the experimental data were subjected to the Scatchard analysis as described in Materials and Methods (Fig. 3b). Least squares analysis of the data gave an average association

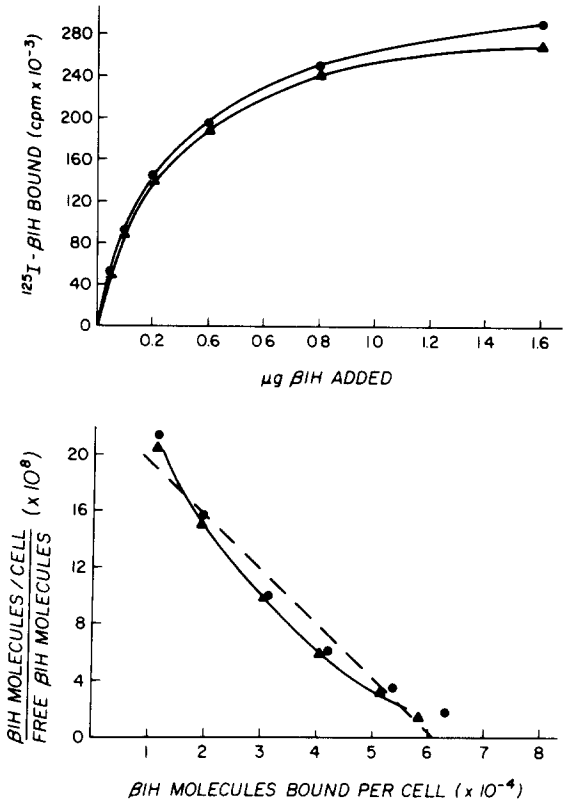

FIG. 3. Quantitative analysis of the interaction of varying concentrations of ^{125}I-β1H with EAC14^{oxy}23. In the upper panel the cpm ^{125}I-β1H bound is shown as a function of the amount of ^{125}I-β1H added; the lower panel is a Scatchard analysis of the same data (see text). In both, total binding to EAC14^{oxy}23 (●) and binding corrected for EAC14^{oxy}2 (▲) are shown. In the lower panel the dashed straight line has been fit by the method of least squares to the corrected data, and the unbroken line is a smooth curve drawn by hand through the same points.

constant (K) of 2.3 × 10^9 L/M for this experiment; the range obtained in five similar experiments was 2–5 × 10^9 L/M. Although the correlation coefficient for a straight line, obtained via the least squares analysis, was greater than 0.9 in all five experiments, the smoothest curve through the data points was always concave towards the abscissa (see Fig. 3b) rather than straight. As is also evident from Fig. 3b, the nonlinearity is not due to β1H binding to EAC14^{oxy}2, since correction for this binding does not greatly improve the straight line fit. Possible reasons for this deviation from linearity are given in the Discussion.

Extrapolation of the experimental data points in Fig. 3b to the abscissa indicates a maximum of 60–70,000 ^{125}I-β1H molecules bound per cell. In the Scatchard analysis shown in Fig. 4, EAC14^{oxy}2 ^{131}I-C3 were used to determine the number of β1H molecules bound per C3b molecule. Except for the ^{131}I-C3, the experimental conditions were identical to those used for Fig. 3. In Fig. 4, therefore, r represents the number of β1H molecules bound per C3b molecule. A K value of 3.9 × 10^9 L/M was obtained from the slope of the line, and

extrapolation to the abscissa indicates a value for r of 0.5, equivalent to an average of one molecule of β1H per two bound C3b molecules. The range for r in the three experiments done in this manner was 0.5 to 0.8 β1H per C3b; in no experiment was a 1 to 1 relationship achieved.

Influence of Fluid Phase C3 and C3b on the Binding of β1H to Cell Bound C3b. Evidence that interaction between C3b and β1H occurs in the fluid phase is provided by the data in Table I. For this experiment, tubes containing a constant amount (0.1 μg) of ^{125}I-β1H and increasing concentrations of either native C3 or C3b (prepared as described in Materials and Methods) or unlabeled β1H were incubated for 15 min at 30°C with 5×10^6 EAC43 in a total vol of 1 ml. The amount of ^{125}I-β1H bound was determined by the ^{22}Na procedure. Table I compares the concentration of unlabeled β1H required for 50% inhibition of ^{125}I-β1H binding with the amounts of C3 and C3b required for similar inhibition. At relatively high concentrations (approximately 1,000-fold molar excess over β1H), both native C3 and C3b inhibit binding of ^{125}I-β1H to C3b-bearing cells. Contamination of the C3 or C3b preparations with small amounts of β1H does not explain the results shown in Table I, since absorption with anti-β1H conjugated to Sepharose 4B has no effect on their inhibitory capacity. Similarly,

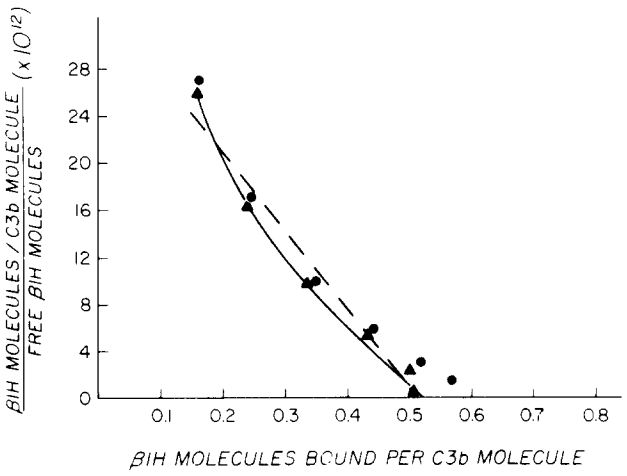

FIG. 4. Scatchard analysis of binding of ^{125}I-β1H to EAC14^{oxy}23 prepared with ^{131}I-C3. The latter allowed the enumeration of the numbers of C3b molecules which was 64,000/cell for this experiment. As in Fig. 4, total binding (●) and that corrected for EAC14^{oxy}2 (▲) binding are given. The dashed and smooth lines are also the same as in Fig. 3.

TABLE I
Inhibition of ^{125}I-β1H Binding to EAC43 by β1H, C3b, or C3

Inhibitor	50% inhibitory concentration	Relative molar inhibitory concentration (50%)*
	ng/ml	
β1H	125	1
C3b	141,250	942
C3	178,000	1,142

* Refers to the molar excess over β1H required for 50% inhibition.

contamination of the native C3 with C3b does not explain their approximately equivalent inhibitory capacity. No alteration in the electrophoretic mobility of the native C3 was seen by immunoelectrophoresis, and a single sharp band corresponding to the C3 α-chain was found when the preparation was examined by SDS-gel electrophoresis under reducing conditions.

Influence of Factor B on the Binding of β1H to Cell Bound C3b. As was the case for C3 and C3b, fluid phase B also inhibited the equilibrium binding of β1H to C3b-coated cells (Fig. 5). For this experiment, 0.1 μg of ^{125}I-β1H was mixed with increasing concentrations of either B, or for comparison, unlabeled β1H. EAC14^{oxy}23 (5×10^6 cells) were then added and after 15 min at 30°C, the amount of ^{125}I-β1H bound was determined by the ^{22}Na procedure. As seen in Fig. 5, B caused a dose-dependent inhibition of ^{125}I-β1H binding to the cells; on a molar basis 280-fold more B than unlabeled β1H was required for 50% inhibition of ^{125}I-β1H binding.

β1H has been previously shown to enhance the decay of factor B from EAC$\overline{43B}$ cells (2, 7). The experimental data shown in Fig. 6 demonstrated that the converse is also true in that B can cause enhanced release of β1H from EAC43 · β1H[2] cells. For this experiment increasing concentrations of B were incubated for 15 min at 30°C with 5×10^7 EAC43 · β1H bearing 4,100 molecules/cell of ^{125}I-β1H. Subsequently, the cells were washed three times with DGVB^{++} and the amount of ^{125}I-β1H remaining bound to the cells was determined. In the absence of B, 12% of the bound β1H was released; over and above this value, the percentage of bound ^{125}I-β1H released was directly proportional to the concentration of B added.

Influence of Properdin on β1H Binding to Cell Bound C3b. In view of the stabilizing effect that properdin has on the interaction between B and C3b (16), it was of interest to examine its influence on β1H binding. EAC43 cells (5×10^6) were incubated for 15 min at 30°C with various amounts of P and 0.1 μg of ^{125}I-β1H. As can be seen from the results shown in Table II, P caused a dose-dependent enhancement of binding of ^{125}I-β1H to EAC43 cells. When an amount of B sufficient to inhibit approximately 50% of the ^{125}I-β1H binding was added, the enhancing effect of P was lost.

In a separate experiment, a Scatchard analysis of ^{125}I-β1H binding to EAC43 cells in the presence and absence of a constant amount of P was performed. Two parallel curves were found, indicating that P did not change the affinity of β1H binding, but rather made more C3b sites accessible to the β1H.

Discussion

The purpose of the present work was to delineate clearly the C3b binding activity of β1H globulin and to investigate the influence that other proteins which bind to C3b have on the interaction of β1H with cell-bound C3b. Previous work had demonstrated agglutinability of EAC43 cells which had been incubated with β1H (8). The fluoresceinated anti-β1H used herein clearly demonstrates that this agglutination was due to bound β1H. Evidence that the β1H is binding to the C3b and not to the cell surface or other complement components is seen in the direct relation between the binding of ^{125}I-β1H, and the amount of

[2] We propose this symbol, EAC43 · β1H to indicate an intermediate bearing bound β1H.

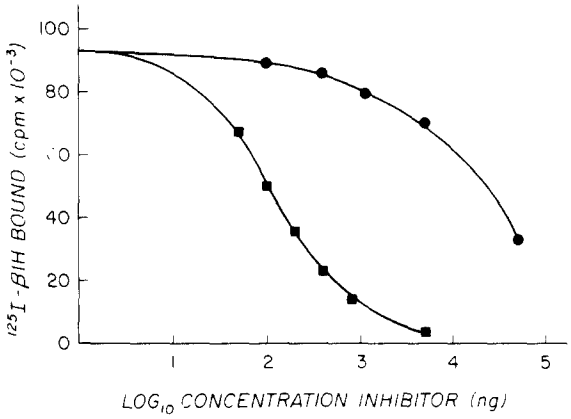

FIG. 5. Inhibition of ^{125}I-βH binding to EAC14^{oxy}23 by unlabeled βH (■) or B (●).

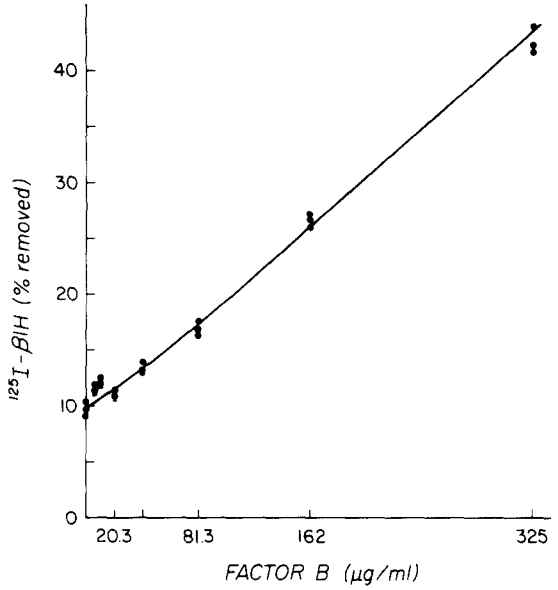

FIG. 6. Removal of ^{125}I-βH from EAC43-βH by varying concentrations of B. Values for triplicate determinations are shown.

C3 used to generate the EAC14^{oxy}23 (Fig. 1). In addition very little ^{125}I-βH binds to the cellular intermediates EA, EAC14, or EAC14^{oxy}2 (Fig. 2).

In the majority of the binding experiments which used ^{125}I-βH, ^{22}Na was used to correct for fluid phase (unbound) βH. This procedure eliminates the necessity of washing and in addition allows the observation of weak binding interactions (24). However, the binding affinity of βH turned out to be quite high; thus little difference was seen in experiments where three washes were used in place of the ^{22}Na procedure (data not shown).

The observation that not all of the ^{125}I-βH was capable of binding to cell bound C3b, even when the C3b was in obvious excess (Fig. 2), was surprising. The most likely reason is simply that the C3b binding site on the βH is somewhat labile, and while antigenically it can be recognized as βH, the C3b

TABLE II
Enhancement of ^{125}I-β1H Binding to EAC43 by Properdin and Its Reversal by Factor B

Agent (μg added)	Molecules ^{125}I-β1H bound per cell	Percent of control
Buffer	7,643	100
P (0.01)	8,053	105.4
P (0.05)	8,537	111.7
P (0.25)	9,568	125.2
P (0.25) + B (40)	3,715	48.6
B (40)	4,069	53.2

binding ability is lost. It does not seem likely that the loss of binding ability is related to the radioiodination procedure; this level of labeling (2–8 × 10^6 cpm ^{125}I/μg β1H) represents an average less than 0.5 atoms of ^{125}I per β1H molecule which is a relatively low level of labeling. Also, competition experiments (Fig. 5 and Table I) indicated that unlabeled β1H was as effective as labeled preparations in binding to C3b. One other possibility to explain the decreased maximum binding would be release of C3b from the cells, and inhibition of β1H binding by the fluid phase C3b such as seen in Table I. However, this would be a viable possibility only if this released C3b was much more effective in inhibition than the trypsin-produced C3b used in Table I.

As discussed by DeMeyts et al. (29), determination of the maximal binding ability of the binding ligand in question is important with respect to further analysis of the binding data. If this is not done, the experimentally determined binding constants will be low (29). For this reason, in the calculation of the free β1H concentrations the total β1H added was adjusted to correspond to this experimentally determined maximal binding value.

The method of Scatchard (25) was chosen to analyze the β1H binding data. This approach has been used in many protein-ligand binding studies and, more recently, in cell receptor-protein binding situations (30, 31). The slope of the line is equal to −K and the intercept at the abscissa is maximum number of binding sites. As stated in Results, the best line through the data points (see Figs. 3a and 4) is concave towards the origin. There are three generally accepted reasons for deviation from linearity in this situation (29): (*a*) The binding site itself is structurally heterogeneous, as are the combining sites of various antibodies directed against the same antigen; the different sites might have different affinities. (*b*) Two or more entirely different classes of binding sites are present, each with different affinity for the ligand. This is frequently observed in experiments in which the amount of nonspecific binding is large. (*c*) Cooperativity between sites may result in changing affinity as sites become occupied. A concave plot is consistent with negative cooperativity (29), in which unfilled sites have a lower affinity for the ligand as increasing numbers of sites become filled.

For the interaction between β1H and C3b, site heterogeneity appears unlikely, since both proteins are supposed to be homogeneous. However, polymorphic forms of C3 are known to exist (32), and β1H has been shown to exhibit some microheterogeneity when subjected to isoelectric focusing (33); thus it is not possible to exclude site heterogeneity as the reason for the nonlinearity of

the Scatchard plots in the present study. The second reason, independent classes of binding sites, has been effectively ruled out, since the second class of sites would be those present on $EAC14^{oxy}2$, and subtraction of the small amount of binding to this intermediate still does not linearize the plots. The final reason, negative cooperativity, appears the most likely: C3b is known to be deposited on the cell surface in a nonrandom manner (34); as the β1H binding sites on the C3b become occupied, the remaining C3b molecules, due to steric reasons, may be less accessible to additional β1H.

The maximum number of binding sites, n, may be underestimated when a concave Scatchard plot such as observed for the interaction of C3b and β1H is extrapolated in a linear fashion. Thus it is possible that the ratio of β1H to C3b might approach 1:1 if sufficiently high β1H concentrations were examined.

The functional consequences of the interaction between C3b and β1H in the fluid phase have been demonstrated by Fearon and Austen (35). Only a small amount of turnover of C3 and factor B was observed in mixtures of C3, B, D, C3bINA, and β1H at concentrations similar to those found in serum. However, if β1H was left out of the reaction mixture both C3 and B were rapidly converted to hemolytically inactive components. Pangburn et al. (4), suggested that β1H was absolutely essential for C3bINA activity on C3b in fluid phase reactions. The inhibitory activity of native C3 and C3b on β1H binding seen in Table I is further evidence of the fluid phase interaction of β1H with C3 and C3b. In spite of the above, direct demonstration of complex formation between C3b and β1H in the fluid phase has not yet been achieved.

When examined for their effect of β1H binding to cell bound C3b, the two other C3b binding proteins, B and P, have essentially opposite effects. B both displaces bound β1H (Fig. 6) from the cell and inhibits the equilibrium binding of β1H to C3b (Fig. 5). This suggests that factor B and β1H interact with the same or closely adjacent sites on the C3b molecule. Others have attributed the ability of substances to activate the alternative pathway to their furnishing a protective "microenvironment," in which C3b bound to their surfaces is less accessible to inhibition by β1H (10, 35, 36). Since the interaction of B with C3b on these same surfaces is supposedly undiminished, these data suggest that B and β1H interact with different sites on C3b. There is no obvious explanation for this apparent paradox.

In the absence of B, P appears to increase the availability of C3b sites for β1H binding. It is evident from the work of Fearon and Austen (16) that P increases the hemolytic activity of factor B. This increase has been attributed to stabilization of the alternative pathway convertase (16), but properdin may cause increased equilibrium binding of B as well. The ability of properdin to extend the half-life of bound β1H is currently being investigated.

Summary

Purified β1H globulin (β1H) was shown to bind to C3b coated cells by both immunofluorescent and radioactive tracer techniques. With EAC43, the amount of β1H bound was directly proportional to the amount of C3 used to prepare the cells; EA, EAC14 and $EAC14^{oxy}2$ bound very small amounts of β1H. The C3b binding site on β1H was labile in that not all of the purified ^{125}I-β1H was

capable of binding to C3b, even when an excess of cell-bound C3b was present. Scatchard analysis of binding of β1H to C3b-coated cells indicated an equilibrium constant of 10^9 L/M. Deviations from linearity were regularly found on Scatchard analyses. This was consistent with the hypothesis that the β1H binding sites exhibit negative cooperativity in that as more sites become occupied, it becomes more difficult to fill the remaining sites. The stoichiometry of the reaction between C3b and β1H was examined using $EAC14^{oxy}23$ prepared with ^{131}I-C3 and β1H labeled with ^{125}I. Between 0.5–0.8 β1H molecules were bound per C3b molecule.

Other alternative pathway components influenced the binding of ^{125}I-β1H to cell bound C3b. Both C3b and native C3 inhibited binding of labeled β1H at an efficiency approximately 1/1,000 that of unlabeled β1H. Factor B inhibited binding with 1/280 the efficiency of unlabeled β1H. Properdin caused a dose-dependent increase in the binding of β1H; this enhancement was abrogated if B was also present in the reaction mixture. Scatchard analysis indicated that the enhancement of β1H binding by P resulted in an increased number of available binding sites rather than an increase in the affinity of binding.

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