

Quantitation of C3 subcomponents on red cells coated with complement *in vitro*

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SUMMARY In order further to characterise and evaluate the reproducibility of human red cells coated with complement *in vitro*, the number of molecules of C3 subcomponents/red cell were determined by Scatchard analysis of equilibrium concentrations of bound and free antibody using ¹²⁵I-labelled goat anti-rabbit IgG. A 1:1 combining ratio was assumed. Red cells coated via the classical pathway had twice as much bound C3b and C3d as alternative pathway-coated cells. Assays using different anti-C3d sera gave different amounts of bound antigen, but results with any one antiserum versus one cell type were reproducible. Anti-C3d sera raised to C3d-trypt and to C3d-KAF detected significantly different amounts of bound C3d on the same cells. Both trypsinisation and serum KAF treatment of classical pathway-coated cells resulted in marked reduction of C3b molecules/cell (over 90% in both cases). Similar reduction in bound C3b was seen after trypsinisation of alternative pathway-coated cells, but serum KAF treatment of such cells had no significant effect. K_0 values were lower with anti-C3c than with anti-C3d. Anti-C3d K_0 values with the various cells coated with complement *in vitro* were not statistically different (approximately 10^7 litres/mol), with the exception of trypsinised alternative pathway-coated cells (approximately 10^8 litres/mol, the same order of magnitude observed with cells coated with C3d *in vivo*). A non-linear relationship between antiglobulin titre and antigen strength was observed. The minimal number of C3d molecules/red cell detectable by agglutination with the various anti-C3d sera ranged from 200 to 670 molecules. The minimal number of C3b molecules detectable by agglutination was approximately 9000 molecules/cell.

Antiglobulin sera for the detection of complement on circulating red cells should contain anti-C3d.¹ Although recent studies^{2,3} have emphasised the need for standardisation of anticomplement antiglobulin sera, there are as yet no accepted standards of performance and potency for anti-C3d reagents. Methods for the preparation of potent antisera specific for complement subcomponents, including C3d, have recently been published, and these antisera can be quantitated in terms of μg specific anti-complement antibody per ml.⁴ Hence standard antiglobulin sera, with known concentrations and equilibrium constants, can be prepared.

A variety of methods for preparing red cells coated *in vitro* with one or more of the subcomponents of C3 and C4 have recently been described.⁵⁻⁷ Such cells are important in the evaluation of antiglobulin sera for monospecificity. The cells are

important also in evaluating the potency of an antiglobulin serum and as control cells for quality assurance of a predictable level of performance of the antiserum. Appropriate control cells require standardisation of the amount of antigen present on the cell surface. Achievement of this goal requires that the amount of the specified antigen bound to the red cells can be reliably quantified. Such quantitation of the complement content on the standard test cells is necessary to ensure reproducibility and accuracy of the standardisation methods for antiglobulin sera and antiglobulin testing. This will allow for reproducibility of results within a given laboratory and valid comparison of results among different laboratories.

This study is directed to quantification of the number of antigenic molecules/cell on red cells coated with subcomponents of C3 *in vitro*. Since particular quality control situations may need red cells coated with large or with small amounts of

Received for publication 28 February 1980

complement, cells coated with differing amounts of complement were assessed. The reproducibility of such control cells and the relationship of the number of complement subcomponent molecules on the cell surface to antiglobulin reactions are considered. Anti-C3d sera prepared to different C3d immunogens are compared.

Material and methods

CELLS

Blood from the same two healthy donors was used throughout. Human red blood cells (RBC) were coated with complement subcomponents by two previously described methods: (a) LIS cells (E-C4b/3b) were coated with complement via the classical pathway by incubation in low-ionic-strength sucrose solution at 37°C,⁵ and (b) E-C3b(A-P) were coated via the alternative pathway in a sucrose-Mg⁺⁺-EDTA medium at 0°C.⁷ Both LIS and E-C3b(A-P) cells were treated with trypsin⁵ or with serum KAF (Konglutinogen Activating Factor; C3b inactivator)⁷ to convert bound C3b to C3d. Four types of C3d-coated cells were thus available: LIS-tryp, LIS-KAF, E-C3b(A-P)-tryp, and E-C3b(A-P)-KAF.

ANTISERA

Six rabbit anti-human C3d sera were used. The methods of preparation, characteristics, and antibody concentrations of these antisera have been described elsewhere.⁸ Briefly, R101-R104 were made by immunising rabbits with well-washed, homogenised precipitin lines obtained by reacting anti-C3d with the low-molecular-weight fractions derived from either KAF treatment (R101 and R103) or trypsin treatment (R102 and R104) of purified C3-C3b. R87 was obtained using a purified, soluble, low-molecular-weight fraction of trypsin-treated C3-C3b as immunogen. For R84, rabbit red cells coated with human C3 in the presence of Mg⁺⁺-EDTA and then trypsinised were used as the immunogen. Rabbit anti-human C3c was purchased from Hoechst-Behringwerke Pharmaceuticals, Montreal, and contained 5731 µg antibody/ml.

Goat anti-rabbit IgG was purchased from Biotek Laboratories, St Louis, Mo, USA, and was purified and labelled with ¹²⁵I as previously described.⁴ It was used at a concentration of 100 µg/ml.

METHODS

Standard serological techniques⁹ were used throughout. Titre scores were determined by summing the agglutination reactions obtained with serial dilutions of a potent anti-C3d serum as follows: 4+ = 10, 3+ = 8, 2+ = 5, 1+ = 3, ½+ = 2.

Quantitation of C3 subcomponent molecules/RBC

A modification of the method described by Rochna and Hughes-Jones¹⁰ was used. All antisera were previously neutralised with purified human IgG to prevent cross-reactivity with any IgG on the red cells. Anticomplement sera were serially diluted in 1% bovine serum albumin/phosphate buffered saline (BSA/PBS) at concentrations designed to achieve antibody excess; 0.4 ml of each dilution of antiserum was added to 0.1 ml aliquots of 5% complement-coated cell suspensions, and the mixture was incubated, with mixing, at 22°C for 30 minutes. The cells were then washed four times in ice-cold 1% BSA/PBS, and to each tube was added 0.4 ml (40 µg) ¹²⁵I-labelled goat anti-rabbit IgG. The mixture was again incubated with mixing at 22°C for 30 minutes. Using a microfuge (Eppendorf centrifuge 5412), the cells were sedimented at 15 000 rpm through *N*-butyl phthalate, and the button of RBC-bound ¹²⁵I-goat antibody was counted in a gamma-counter. Non-complement-coated, EDTA-treated normal RBC, as a negative control, were treated concurrently in the same way, and the non-specific counts were subtracted. Bound ¹²⁵I-radioactive counts were converted to µg antibody bound using a calibration curve as previously described.⁴ $\frac{[\text{Bound antibody}]}{[\text{Free antibody}]}$ was plotted against

[bound antibody] and, following the principle of Scatchard analysis,¹¹ extrapolation of the curve to the base-line gave the maximum binding of the antibody. Since 1 mol/l = 6 × 10²³ molecules (Avogadro's number), the number of molecules/litre can be calculated. Since the number of RBC present and the volume are known, the number of molecules/RBC can be calculated. A 1:1 combining ratio was assumed. The K₀, or equilibrium constant, values were determined by the Karush plot.¹²

Results

QUANTITATION OF RED CELL BOUND C3 SUBCOMPONENTS USING VARIOUS ANTI-C3d

Previous studies have suggested that LIS-tryp, E-C3b(A-P)-tryp, and LIS-KAF cells have only the C3d subcomponent of C3 on their surfaces. LIS cells, E-C3b(A-P)-KAF cells, on the other hand, are thought to have C3d in addition to C3b on their surfaces. Figure 1 shows the results of more than 100 determinations of the number of molecules of C3 subcomponents/RBC on red cells prepared in different ways using the described six anti-C3d antisera. The results in the figure represent data obtained using all six antisera. In the combined antisera data, with any one type of cell a wide variation in the number of C3 molecules detected was

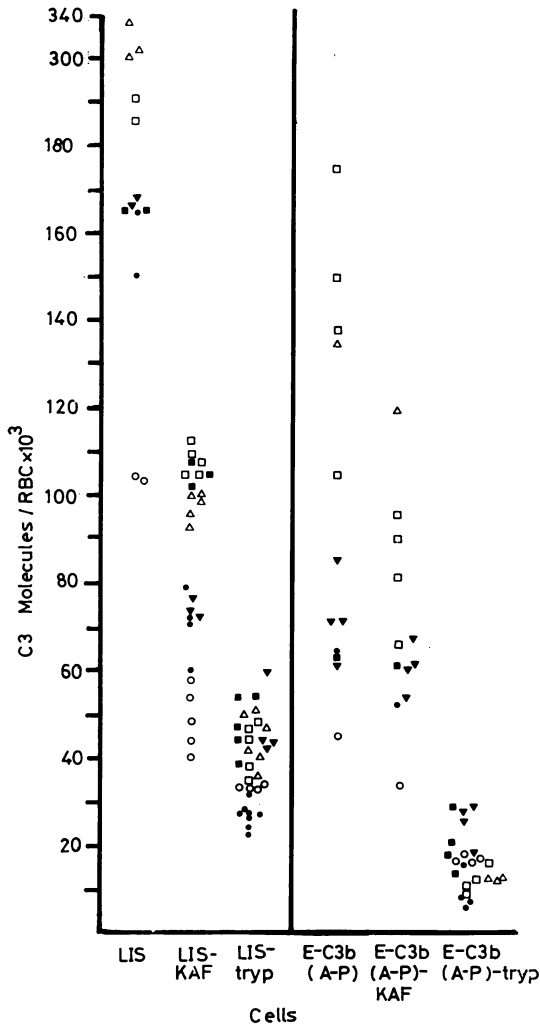


Fig. 1 Combined results of determinations of C3 subcomponent molecules/RBC on cells coated with complement in vitro by various methods. (Different symbols represent different anti-C3d sera: □ = R101, ■ = R102, △ = R103, ▼ = R104, ○ = R84, ● = R87.)

observed. The overall average within variation (one antiserum with one cell type) was 4970 molecules/RBC.

The anti-C3d sera were prepared to different C3d immunogens in order to determine differences in specificity of anti-C3d-try versus anti-C3d-KAF. The results in the Table indicate that such differences in specificity do exist. All antisera were tested against one type of cell on the same day under the same conditions; alternatively, on other occasions, one antiserum was tested against all types of cells. While

R101 and R103 (made to the C3d-KAF antigen) appeared to detect fewer molecules/RBC on LIS-try cells than did R102 and R104 (raised to the C3d-try antigen), the difference is not statistically significant. With E-C3b(A-P)-try cells, however, the antisera raised to the C3d-KAF antigen (R101 and R103) did detect significantly fewer molecules/RBC than did R102 or R104 (raised to the C3d-try antigen), $p < 0.01$. With E-C3b(A-P)-KAF cells, the antisera raised to the C3d-KAF antigen detected significantly more molecules/RBC than did antisera raised to C3d-try, $p < 0.001$. With LIS-KAF cells, there was no statistical difference between the number of molecules detected by R102 or R103, but R101 ('pure' anti-C3d-KAF) detected significantly more molecules/RBC than did R104 ('pure' anti-C3d-try), $p < 0.001$. With LIS cells and with E-C3b(A-P) cells, significantly more molecules/RBC were detected by R101 than by R104, in both cases $p < 0.001$.

While both R84 and R87 were made to the C3d-try antigen, different immunogens were used in each case. R84 consistently detected a higher number of molecules/RBC on LIS-try and E-C3b(A-P)-try cells than was observed with R87; the converse was true for LIS-KAF and E-C3b(A-P)-KAF cells.

EFFECT OF TRYPSIN AND OF SERUM KAF ON BOUND C3b AND C3d

Figure 2 shows the mean average C3 sites on the various complement-coated cells as determined with anti-C3c and with R101 and R104 anti-C3d reagents. LIS cells were found to have significantly more detectable C3d subcomponent molecules/RBC on

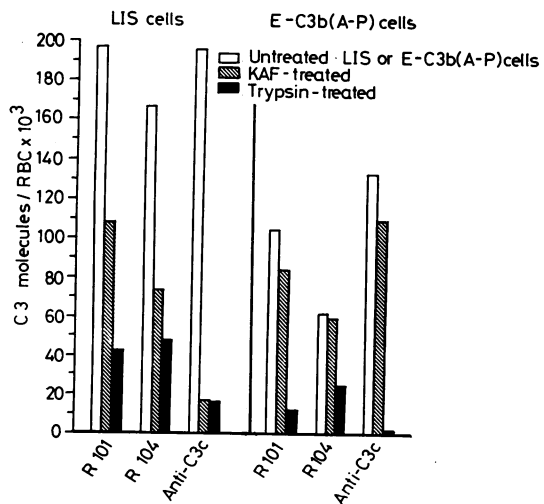


Fig. 2 Effect of trypsin and of serum KAF on red cell bound C3b and C3d.

Comparison of anti-C3d-KAF (R101 and R103) and anti-C3d-tryp (R102, R104, R84, and R87) on determinations of red cell bound C3d-KAF and C3d-tryp

Antiserum	Mean average molecules/RBC \pm 1 SD			
	LIS-tryp cells	E-C3b(A-P)-tryp cells	LIS-KAF cells	E-C3b(A-P)-KAF cells
R101	42 880 \pm 5319 (5)*	12 125 \pm 3112 (4)	108 060 \pm 2880 (5)	83 250 \pm 11 299 (4)
R102	47 600 \pm 5817 (5)	20 250 \pm 4941 (4)	105 000 \pm 2449 (3)	60 750 (1)
R103	44 333 \pm 5280 (6)	12 413 \pm 426 (3)	97 620 \pm 2760 (5)	120 000 (1)
R104	47 250 \pm 7395 (4)	25 000 \pm 4301 (4)	73 750 \pm 1541 (3)	60 250 \pm 4802 (4)
R84	33 250 \pm 433 (4)	16 875 \pm 739 (4)	48 920 \pm 6513 (5)	33 750 (1)
R87	27 250 \pm 4115 (8)	12 940 \pm 1766 (4)	70 375 \pm 6794 (4)	52 500 (1)
All antisera combined results	39 325 \pm 9608 (32)	16 783 \pm 5778 (23)	81 990 \pm 23 697 (25)	68 416 \pm 22 282 (12)

*Figures in parentheses = number of determinations.

their surfaces than did LIS-KAF cells, $P < 0.05$. LIS-tryp cells had fewer detectable C3d molecules/RBC than did LIS-KAF cells, $P < 0.01$. The difference between the number of detectable C3d subcomponent molecules/cell on E-C3b(A-P) and E-C3b(A-P)-KAF cells was not statistically different, but E-C3b(A-P)-tryp cells had significantly fewer C3d molecules on their surfaces, $P < 0.001$. The above statistical results applied with all six anti-C3d sera.

Overall, more C3 subcomponent molecules were bound to LIS cells than to E-C3b(A-P) cells. Whereas LIS cells showed a marked reduction in C3b molecules/cell on treatment with KAF and with trypsin (over 90% reduction in both cases), E-C3b(A-P) cells, although showing marked loss in C3b molecules/cell on trypsinisation (99% reduction), had only slight reduction in C3b molecules/cell after serum KAF treatment (approximately 22% reduction). The reduction in C3b molecules/cell after KAF treatment of E-C3b(A-P) cells was no different when the cells were incubated in saline in place of serum KAF and probably represents nonspecific degradation of C3b. Although a greater number of molecules/RBC were detected on the LIS-tryp cells than on the E-C3b(A-P)-tryp cells, the titre or titre scores of the cells with anti-C3d tended to be slightly higher with E-C3b(A-P)-tryp cells. This is probably due to the higher K_0 values obtained with the latter cells.

EQUILIBRIUM CONSTANTS

The K_0 values of anti-C3d with red cells coated with C3 subcomponents in various ways are shown in Figure 3. Mean K_0 was $3.7 \pm 4.4 \times 10^7$ litres/mol. There were no significant differences between the K_0 values of individual anti-C3d sera against any or all types of cells. The K_0 values with the various *in vitro* coated cells were not statistically different, with the exception of E-C3b(A-P)-tryp cells which had a higher K_0 than all other *in vitro* coated cells tested ($P < 0.05-0.01$). The mean average K_0 for E-C3b(A-P)-tryp cells was of the order of 10^8

litres/mol, the same order of magnitude as seen with red cells coated with C3d *in vivo*. The K_0 values of the anti-C3c serum with the *in vitro* coated cells were lower than those observed with anti-C3d. In general, the K_0 value for anti-C3c with LIS-derived cells was of the order of 10^5 litres/mol, and with E-C3b(A-P)-derived cells, 10^6 litres/mol.

CORRELATION OF ANTIGEN CONCENTRATION WITH ANTIGLOBULIN TITRES

Figure 4 shows the relationship between antiglobulin titre score and the number of molecules of C3d/RBC. The results in the figure are from determinations with one anti-C3d serum (R103) against a variety of *in vitro* and *in vivo* C3d-coated red cells. Cells were coated *in vitro* with varying amounts of complement on their surfaces by varying the amount of complement (fresh normal serum) during cell preparation. The regression equation ($y = 2.462 + 0.0497x$, α (intercept) is in the range 2.380-2.544 with a 95% confidence level; β (slope) is in the range 0.044-0.055 with a 95% confidence level. The coefficient of correlation ($r = 0.917$, $P < 0.001$). It is evident that correlation is good and, at high antigen strength, the relation between antiglobulin titre and antigen strength is non-linear. The results with this particular antiserum indicate approximately 325 C3d molecules/RBC to be present on the cell surface for a titre score of 1 (trace agglutination) or approximately 375 C3d molecules/RBC for a titre score of 2 ($\frac{1}{2}$ + agglutination). Although similar relationships were seen with different anti-C3d sera, the slopes were different in each case. The minimal number of C3d molecules/RBC detectable by agglutination with various antisera ranged from 200 to 670 molecules/RBC.

For anti-C3c, a less steep slope of the relationship of titre score to molecules/cell was found than was observed with anti-C3d sera. The minimal number of C3b molecules/RBC detectable on LIS cells by agglutination with anti-C3c was 9400 ± 3250 C3b molecules/RBC.

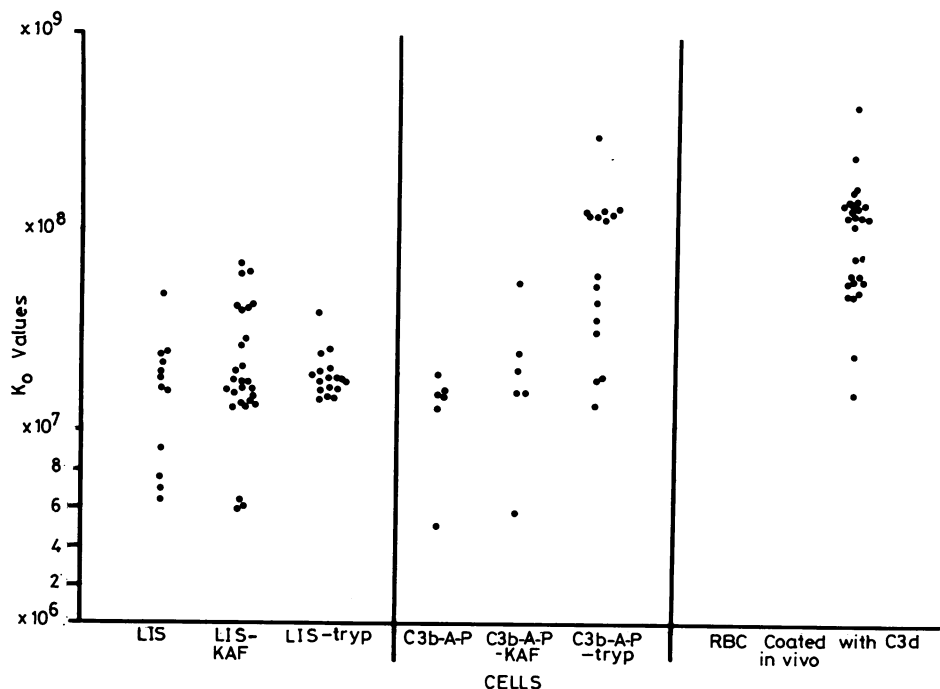


Fig. 3 K_0 values of anti-C3d sera with complement bound to red cells in vitro and in vivo.

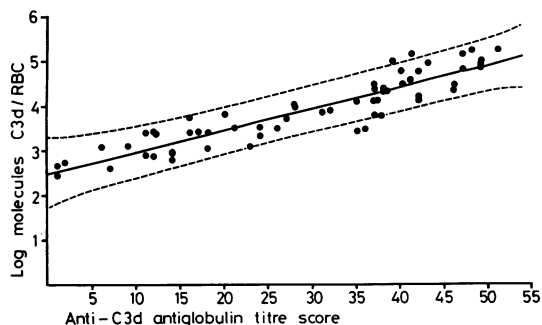


Fig. 4 Correlation of antiglobulin titre score and antigen strength.

Discussion

The present report is concerned with the quantitative assessment of control human red cells coated *in vitro* with C3 subcomponents, C3d in particular.

Immunochemical methods for the measurement of fluid phase C3 cannot be used for bound C3, and generally available techniques for detecting bound C3, for example, the antiglobulin test, are not quantitative. Although quantitation of the amount of fixed complement has been difficult, a few techniques have been developed. Logue *et al.*¹³ used the

C1 fixation and transfer test to study C3 in patients with autoimmune haemolytic anaemia. Although useful in a model system, the test is indirect and time-consuming. Borsos and Leonard¹⁴ developed an anti-C3 inhibition assay which was used by Fischer *et al.*¹⁵ to determine the number of molecules of C3 on human red cells. They were able to show a correlation between the antiglobulin titration score and the amount of bound C3 per red cell. The authors noted that, for cells coated *in vivo*, the presence or absence of haemolysis was not explained by variations in the amount of IgG on the cells but could be roughly correlated with the amount of complement on the cells. Thus, the amount of C3 bound to a red cell appears to be an important determinant of haemolysis *in vivo*. The *in vitro* coated cells studied by Fischer *et al.* were sensitised via antibody. Low-ionic-strength media are more often used to prepare complement-coated control cells for routine use in the laboratory; the present study examines such cells.

A quantitative assay for bound complement should allow comparison of the amount of bound C3 with results obtained by conventional serological techniques. The previous work on quantitation of bound complement components has all been done with 'whole C3' and insufficiently characterised 'anti-C3' sera. It is now well recognised that it is the presence

of the C3d subcomponent of C3 on the red cell membrane that indicates that an immune reaction has occurred *in vivo*. The applicability of the results with 'C3' to the biologically important C3d subcomponent needs to be investigated.

In order to quantitate the number of C3b and C3d molecules on erythrocytes, a method utilising radiolabelled antibody was used. The method is a modification of that described by Rochna and Hughes-Jones¹⁰ for the quantitation of D antigen sites on red cells and subsequently used for quantitation of other red cell antigens. The method is based on the law of mass action using an antibody excess; the results are derived from Scatchard analysis. This method appears to be a more direct method of quantitation than the C1 fixation and transfer method or the C3 inhibition assay. By using Scatchard analysis of results, different K_0 values of the antisera are taken into account and do not affect the estimates of the antigen concentration. Once the antisera are prepared, the method is relatively simple. Since the method using ¹²⁵I-labelled anti-rabbit IgG to determine bound anti-C3d will measure all rabbit IgG on the test cells, only antisera specific for C3 subcomponents were used. The high values observed with strongly C3b-coated red cells with R101 and R103 probably reflect the presence of both anti-C3c and anti-C3d in these reagents. When strongly complement-coated cells were assayed, the uptake of labelled anti-rabbit IgG by the cells was more than 50-fold above the non-specific uptake by control cells, and reproducibility of measurements was good. When weakly coated cells were used, the ratio of specific to non-specific uptake of labelled antiglobulin was, at times, less than twofold, and a somewhat greater variation between repeated measurements was observed.

The accuracy of the method is dependent on the accurate determination of the specific activity of the IgG fraction containing anti-C3d, and the assumption is made that the anti-C3d molecules had the same average specific activity as all the IgG molecules. Since the combining ratio of anticomplement and bound C3 subcomponents is not yet known, in these experiments the binding ratio has been taken as 1:1.

It has recently been shown¹⁶ that soluble C3d prepared by treatment of C3-C3b with KAF differs in a number of ways from C3d prepared by treatment of C3-C3b with trypsin. The present study indicates subtle differences in the anti-C3d reactivity of antisera raised to C3d-KAF and C3d-tryp. As would be expected, the results indicate that an antiserum reacts best with the antigen against which it was raised. It appears that apparently small differences in immunogen structure can influence the specificity

of the antibody.

The Scatchard curves obtained from the assays were curvilinear, indicating heterogeneity of the binding constants of anti-C3d within a sample. There were no significant differences within the six anti-C3d sera in the K_0 against any or all types of cells. Thus, differences in numbers of molecules/RBC obtained using different antisera could not be attributed to variation in the equilibrium constants.

It is evident from Fig. 2 that with LIS cells, trypsinisation causes greater degradation of C3b to C3d than does treatment of the cells with KAF. With both LIS and E-C3b(A-P) cells, trypsinisation did not completely convert C3b to C3d. With LIS cells, a mean of $17\,100 \pm 4392$ C3b molecules/RBC remained. Trypsinisation of E-C3b(A-P) cells was more effective, leaving a mean of only 2300 ± 2500 C3b molecules/RBC. Both LIS-tryp and E-C3b(A-P)-tryp cells have previously been considered to have only the C3d subcomponent of C3 on their surfaces, as determined by agglutination tests with monospecific anti-C3c sera used at a concentration of $100\ \mu\text{g/ml}$. Increasing the concentration of the anti-C3c sera to $500\ \mu\text{g/ml}$ resulted in positive agglutination tests with both LIS-tryp and E-C3b(A-P)-tryp cells ($1+$ and $\frac{1}{2}+$ agglutination, respectively). Increasing the time of trypsinisation up to threefold did not further reduce the number of C3b molecules on the cells. The findings suggest a population of C3b molecules bound to red cells resistant to the action of trypsin.

The resistance of E-C3b(A-P) cells to serum KAF treatment has been discussed elsewhere⁷ and is probably due to the presence of Bb and activated properdin on the cell surface.

Chaplin¹⁷ showed that the distinction between 'strongly' and 'weakly' coated RBC could be assessed to only a limited extent by the strength or weakness of the antiglobulin test reaction. The results presented in Fig. 4 confirm Chaplin's observation and emphasise that this is particularly true for strongly coated cells.

The results indicate that the described *in vitro* complement-coated red cells can be prepared and quantitated with reasonable reproducibility. Although anti-C3d sera may appear similar by immunoprecipitation techniques, differences in specificity do exist, depending on the method used to prepare the C3d immunogen. In general, anti-C3d sera raised to the C3d-KAF antigen are better for detecting C3d-KAF cells (and probably red cells coated with C3d *in vivo*), whereas antisera raised to the C3d-tryp antigen are better for detecting cells coated with the C3d-tryp antigen (eg, *in vitro* coated control cells). The results suggest that E-C3b(A-P)-tryp cells may be closest to red cells coated with C3d *in vivo* and

may be the control cell of choice for assessing anti-C3d sera.

Only two donors were used, and preliminary experiments do indicate significant differences when other donors are used or when infection or subclinical disease is present in the donors. Blood used for the preparation of control cells should, therefore, be from a regular, healthy donor to allow comparison with previous evaluations.

It is hoped that such methods will allow for the development of standard cell suspensions sensitised with known amounts of complement subcomponents, C3d in particular, and of reference antisera, of known concentration, standardised against the cells.

The investigation was supported by Ontario Ministry of Health grant PR658-C and a grant (MA-6693) from the Canadian Medical Research Council.

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