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DETERMINATION OF ALTERNATIVE PATHWAY OF COMPLEMENT ACTIVITY IN MOUSE SERUM USING RABBIT ERYTHROCYTES

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Rabbit, mouse and sheep erythrocytes expressing different concentrations of membrane sialic acid were used to study possible modes of activation of the alternative complement (C) pathway in mouse, human and guinea pig serum. Mouse erythrocytes activated only human serum, whereas rabbit erythrocytes activated the sera of all three species. Based on the observation that rabbit erythrocytes activate the murine alternative C pathway a method for estimation of alternative C pathway activity (AP50 value) in mouse serum was devised analogous to that used for human AP50 determination. The method is not very sensitive to ageing or to batch variation of the indicator cells. The AP50 value of mouse serum measured by this method is of the same order as for human and guinea pig serum. Mouse serum AP50 activity is partly determined by natural anti-rabbit erythrocyte antibodies and is sensitive to heating (15′ at 48°C and 4′ at 56°C), and to the actions of cobra venom factor, zymosan and cysteine. Strain and sex differences with respect to AP50 activities of mouse sera were observed.

INTRODUCTION

For the determination of the classical complement (C) pathway activity in the sera of different animal species a sheep erythrocyte-antibody (EA) complex is commonly used (Mayer, 1961). Sera of some animals, including mice, fail to lyse EA (Brown, 1943). Mouse serum nevertheless forms EAC complexes in which the presence of C_3 may be demonstrated by rosette formation with B lymphocytes (Bianco et al., 1970), which indicates the presence of a C system at least as far as C_3. The inability of mouse serum to lyse EA might be due to a defect in one of the late C components, e.g. C_5 as suggested by Berden et al. (1978), or to the presence of some potent C-associated inhibitory system (Adolphs, 1973) with the C_{3b} inactivator system being the most probable candidate (Ranken and Linscott, 1979). The C_{3b} inactivator system of other animals has been shown to consist of C_{3b} inactivator (C_{3b}INA) and β_1H (Pangburn et al. 1977; Harrison and Lachmann, 1978).

Non-sensitised erythrocytes with low membrane sialic acid concentrations such as those of mice (MRBC) and rabbits (RRBC) activate the alternative

pathway of human C (Platts-Mills and Ishizaka, 1974; Czop et al., 1978), whereas sialic acid-rich sheep erythrocytes (SRBC) do not (Fearon, 1978). The mechanism of alternative C pathway activation by MRBC, RRBC and sialase-treated SRBC seems to be protection of membrane-bound alternative pathway C_3 convertase against decay-dissociation by the C_{3b} inactivator system (Fearon and Austen, 1977; Fearon, 1978; Kazatchkine et al., 1979).

Since mouse C is not likely to be activated by non-sensitised isologous MRBC, this study deals with the possible ability of RRBC to escape the potent regulatory influences of the C_{3b} inactivator system in mouse serum. With a system similar to that described for estimation of the human alternative C pathway (AP50) activity (Takada et al., 1978) the AP50 activity of mouse serum was determined using RRBC as indicator cells. Values thus obtained have been compared with those using MRBC and SRBC and with the AP50 values of human and guinea pig serum in the same test systems.

MATERIALS AND METHODS

Animals

Unless otherwise stated, male F_1 (BALB/c × Swiss inbred) mice, bred and maintained in our laboratory and 2.5—4 months old were used as serum, plasma and MRBC donors. In one experiment female mice were used and in another male mice of both parent strains and of the C3H/HeJ and C3HeB/FeJ (Jackson Laboratories, Bar Harbor, Maine, U.S.A.) strains.

Male New Zealand white rabbits, obtained from the Central Institute of Laboratory Animal Breeding (CPB, Austerlitz, The Netherlands) were used as RRBC donors. Unless otherwise stated erythrocytes of one rabbit were used.

Preparation of serum and plasma

Mice were bled from the retro-orbital venous plexus by means of capillary tubes. After the blood had been allowed to clot for 2 h at room temperature, serum was separated by centrifugation at 4° C and used immediately.

One experiment used mouse EGTA-plasma obtained by mixing 0.96 ml fresh mouse blood with 40 µl of a Mg-EGTA stock solution, pH 7.4. The stock solution contained 0.2 M ethyleneglycol-bis-(2-aminoethyl)-tetra-acetic acid (EGTA) and 0.5 M $MgSO_4$ and was sterilised by millipore filtration.

Freshly collected human and guinea pig serum were stored at —70° C.

Erythrocytes

Blood collected from the ear artery of rabbits and the orbital venous plexus of F_1 mice was diluted in 1 : 1 ratio with Alsever's old solution and

used as the source of RRBC and MRBC. RRBC were regularly used within 5 days after collection, but only fresh MRBC were tested.

Sheep blood, stored in Alsever's old solution, was obtained from the National Institute of Public Health (RIV, Bilthoven, The Netherlands). SRBC were used up to 2 weeks after collection.

To elute possibly adsorbed serum proteins all red blood cells were washed three times with an aqueous 0.16 M sodium iodide solution before use (Najjar, 1974; Fyhrquist and Wallenius, 1975). The concentration was photometrically adjusted to 1.5×10^8 cells per ml buffer used in the test.

Buffers

Veronal saline buffer, concentrated 5 times (VSB-5×), pH 7.4, was prepared as described by Mayer (1961). Veronal buffered saline, pH 7.4, containing 20 mM Mg^{2+} and 8 mM EGTA (EGTA-VB) was prepared by mixing 20 vols of VSB-5× with 4 vols of the Mg-EGTA stock solution as described above and 76 vols of distilled water (Takada et al., 1978). Veronal buffered saline, pH 7.4, containing 10 mM ethylenediamine tetra-acetic acid and 0.1% gelatin (EDTA-GVB) was prepared with VSB-5× and a stock solution containing 0.2 M EDTA (Takada et al., 1978). Veronal saline buffer, pH 7.4, containing 0.15 mM Ca^{2+} and 0.5 mM Mg^{2+} (VSB^{++}) was prepared with VSB-5× and a stock solution of 150 mM Ca^{2+} and 500 mM Mg^{2+} (Mayer, 1961).

Determination of alternative C pathway activity

Two hundred µl of mouse, human or guinea pig serum serially diluted with EGTA-VB were mixed with 100 µl of an erythrocyte suspension containing 1.5×10^8 washed RRBC, MRBC or SRBC per ml EGTA-VB and incubated for 30′ at 37°C (Takada et al., 1978). To stop the reaction 2.2 ml of EDTA-GVB was added, after which the cells were spun down.

The percentage of haemolysis was calculated by the formula:

$$\% \text{ Haemolysis} = \frac{E_{412}(\text{fresh serum}) - E_{412}(\text{heat-inactivated serum})}{E_{412}(\text{water}) - E_{412}(\text{buffer})} \times 100 ,$$

in which E_{412} (fresh serum) represents the extinction of the supernatant at 412 nm, E_{412} (heat-inactivated serum) the E_{412} of supernatant from heat-inactivated (30′ 56°C) serum, E_{412} (water) the extinction of a water-lysed control, and E_{412} (buffer) the 0% lysis control.

Alternative C pathway activity was expressed in AP50 units per ml, in which 1 AP50 unit causes 50% lysis of the test system. In one experiment the incubation time was varied and in another VSB^{++} was used as diluent instead of EGTA-VB.

Absorption of mouse serum with RRBC

One vol of ice-cold packed washed RRBC was mixed with three vols of ice-cold fresh mouse serum and incubated for 20′ at 4° C. After centrifugation at 4° C the supernatant was recovered.

Cobra venom factor treatment of serum donors

Cobra venom factor (CoF) purified according to Ballow and Cochrane (1969) was kindly provided by Dr. W. Seinen, Pathology Institute of the Veterinary Faculty, State University of Utrecht, The Netherlands. Male F_1 mice were intraperitoneally injected twice with 2 units of CoF with an interval of 8 h. The mice were bled for serum 24 h after the first injection.

Treatments of mouse serum

Zymosan treatment was performed by incubating 99 vols of fresh mouse serum with 1 vol of sonicated zymosan for 60′ at 37° C (Mayer, 1961). After centrifugation the supernatant was recovered.

Storage inactivation of fresh mouse serum was studied by freezing at −70° C and thawing 24 h later or by incubation for varying periods at +20°, 37°, 48° and 56° C. Immediately after these procedures the AP50 activities were determined.

Inhibition experiment

L-cysteine HCl (Fluka, Buchs, Switzerland) was used to inhibit AP50 activity (Takada et al., 1978). Fifty µl of a 70 mM solution were added to the incubation volume of 300 µl to give a final concentration of 10 mM.

Statistical analysis

Results were expressed as the arithmetic mean of n duplicate values. The standard error (S.E.) was used to indicate the reproducibility of the method; the standard error of the mean (S.E.M.) for analysis of significance.

RESULTS

AP50 activities of mouse, human and guinea pig serum as measured with RRBC, MRBC and SRBC

Mouse, human and guinea pig serum were tested for alternative C pathway activity using RRBC, MRBC and SRBC as indicator cells. RRBC were able to activate the alternative C pathway of all three sera tested (Fig. 1). The titres of the sera for mouse and human serum were 25 AP50 units per ml

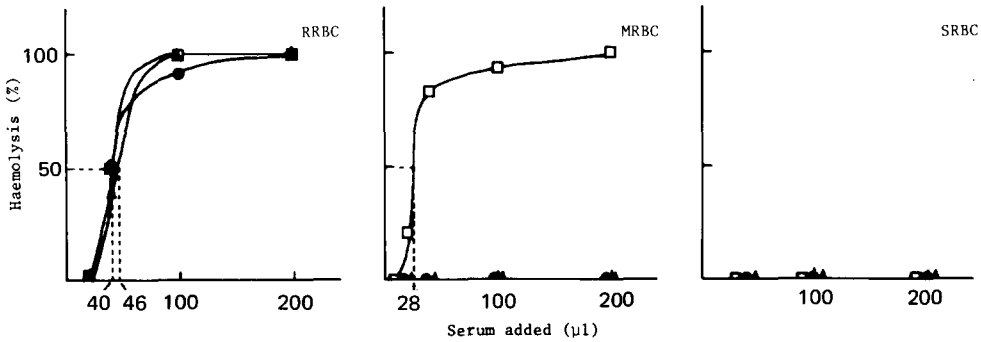

Fig. 1. Alternative C pathway activities of mouse (●), human (□) and guinea pig (▲) serum as measured with three different species target cells.

and for guinea pig serum 21.7 units/ml. MRBC activated the alternative pathway of human complement only, resulting in a titre of 35.7 units/ml, whereas SRBC did not activate the alternative pathway of any of the three sera tested. In further experiments only mouse serum and RRBC were used for AP50 determination.

Reproducibility of mouse serum AP50 values

The AP50 value of mouse serum was estimated with fresh RRBC from 5 different rabbits. As shown in Table 1, AP50 values varied from rabbit to rabbit with extrema of 15.6 and 21.7 units per ml.

The effect of storing RRBC at 4°C on the reliability of mouse AP50 values was investigated, by using one batch of RRBC and mouse serum for homogeneity stored at −70°C. AP50 values decreased slowly with time after RRBC collection (Fig. 2). In further experiments RRBC were used within 5 days after collection.

TABLE 1

Reproducibility of AP50 values estimated with different RRBC.

RRBC donor	AP50 values (units/ml)	Mean	S.E.	S.E.M.
K7962	18.2			
K7963	21.7			
K7964	15.6	18.0	2.3	1.0
K7965	17.2			
K7966	17.2			

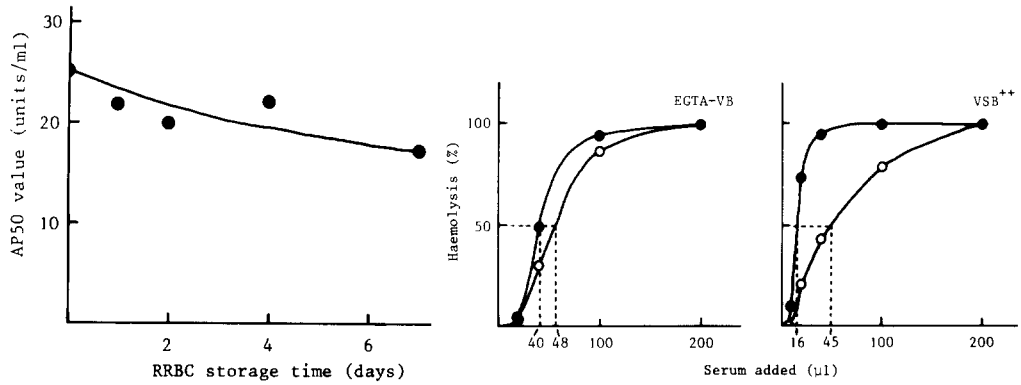

Fig. 2. Effect of ageing of RRBC on the AP50 value of mouse serum.

Fig. 3. Effect of absorption of mouse serum with RRBC (○) on its RRBC-lysing activities in EGTA-VB and VSB^{++}. (●) Represent values for control serum.

Effect of absorption with RRBC on the RRBC-lysing activity of mouse serum

AP50 values of mouse serum before and after absorption with RRBC were determined and compared with values obtained by performing the test in a similar way but with VSB^{++} as diluent instead of EGTA-VB. The AP50 values of normal and absorbed mouse serum were 25 and 20.8 units per ml respectively and for the test in VSB^{++} 62.5 and 22.2 units/ml (Fig. 3).

Effect of variation of incubation time on the AP50 value of mouse serum

The development of the alternative pathway of mouse C activation with time was studied using incubation times of 0, 1, 2, 4, 8, 15, 30, 45 and 60 min. After incubation the test tubes were placed in ice diluted with ice-cold EDTA-GVB and centrifuged at 4°C. Figure 4 shows that lysis by alternative C pathway activation in mouse serum began at 15′, was maximal at about 45′ and decreased thereafter. The decrease between 45′ and 60′ is due to a variably increasing background lysis of the heat-inactivated control initially observable between 30′ and 45′.

Depletion or inhibition of alternative C pathway activity in mouse serum

To obtain mouse serum depleted of alternative pathway components, mice were treated with CoF before bleeding or normal mouse serum was treated with zymosan. As shown in Fig. 5 both treatments resulted in AP50 values below measurable levels (for CoF treatment <2 units per ml and for zymosan treatment <1.5 unit/ml). L-cysteine was added to the incubation

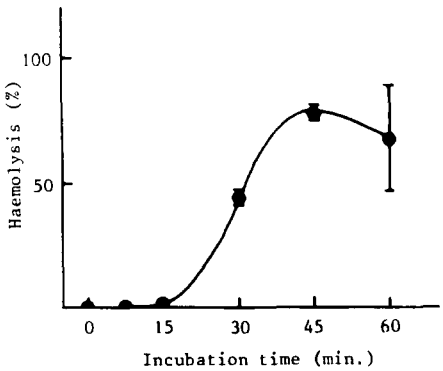

Fig. 4. Development of alternative pathway lysis of RRBC by mouse serum with time.

mixture for mouse AP50 determination. Fig. 5 also shows that the AP50 value of mouse serum in the presence of 10 mM cysteine was 8.0 units per ml compared to a control value of 22.2 units/ml (64% inhibition).

Effects of preincubation of mouse serum at different temperatures on the AP50 value

Mouse serum was frozen at −70°C and thawed 24 h afterwards. Fresh serum of the same batch was incubated for different times at 20°, 37°, 48° and 56°C. After these procedures, the sera were tested for AP50 activities. The freezing and thawing procedure gave a very slight decrease of AP50 activity from 21.7 to 20.6 units per ml (Fig. 6). Curves describing the alternative C pathway activities of mouse serum after incubation at 20° and

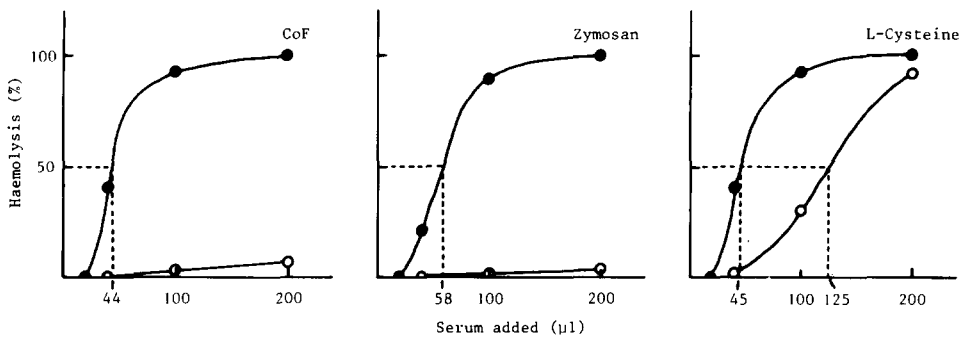

Fig. 5. Effects of depleting or inhibiting reagents on mouse alternative C pathway activity (○). CoF treatment was performed in vivo by injecting mice with 2 × 2 units; zymosan treatment of mouse serum was carried out in vitro by incubating with 1% zymosan; whereas L-cysteine (10 mM) was added to the test tubes for AP50 determination. (●) Represent the values for control serum.

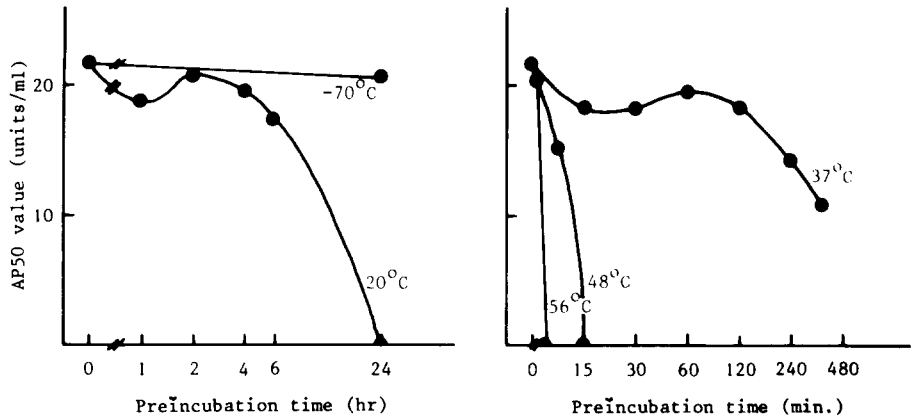

Fig. 6. Sensitivity of mouse AP50 values to storage at different temperatures.

37° C showed intermediate minimum values at 1 h and between 15′ and 30′ respectively. After 6 h of incubation at 20° C the AP50 of serum had decreased to 17.4 units per ml, and after 24 h it had completely disappeared; the latter could, however, be due to infection. Incubation for 3 h at 37° C decreased the AP50 value to 10.9 units per ml, while 15′ at 48° C and 4′ at 56° C sufficed for complete destruction of alternate pathway activity in mouse serum.

Plasma and serum AP50 activities of male and female mice

Mg-EGTA plasma and serum were prepared from the blood of male and female mice and the AP50 values determined. The plasma AP50 values

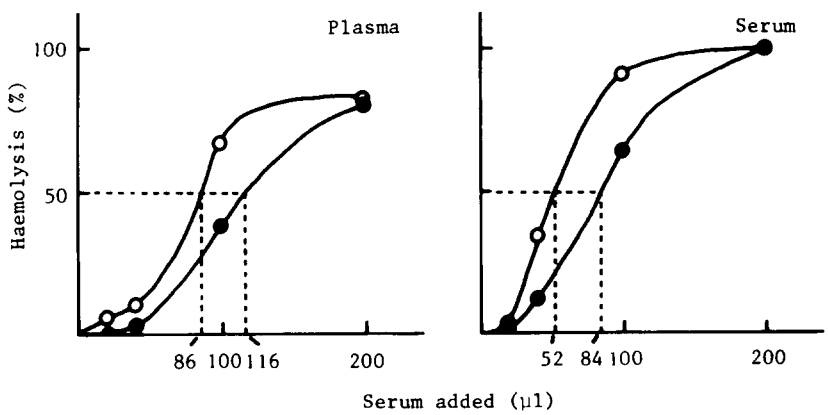

Fig. 7. Comparison of plasma and serum alternative pathway activities of male (○) and female (●) mice.

TABLE 2

Mouse strain dependence of AP50 activity.

Mouse strain	AP50 value (units/ml)
BALB/c	25.0
Swiss inbred	12.4
F_1 (BALB/c × Swiss inbred)	21.9 ± 1.1 (n = 7)
C3H/HeJ	16.1
C3HeB/FeJ	11.5

for male and female mice were 11.6 and 8.6 units per ml and the serum values 19.6 and 11.9 units/ml (Fig. 7).

Strain differences in AP50 activities

The AP50 activity of F_1 (BALB/c × Swiss inbred) mouse serum was compared with that of males of both parent strains and with that of C3H/HeJ and C3HeB/FeJ mice. Inter-strain differences were noted (Table 2). BALB/c and F_1 mice had high, C3H/HeJ mice intermediate, and C3HeB/FeJ and Swiss mice low AP50 activities.

DISCUSSION

Our results show that incubation of RRBC with mouse serum leads to lysis of the cells. As lysis occurs in an EGTA milieu (Fig. 1) and is prevented by CoF injection of serum donors and by zymosan treatment of normal mouse serum (Fig. 5), it is concluded that lysis can be ascribed to alternative C pathway activation. The 64% inhibition of the lytic activity of mouse serum by 10 mM cysteine (Fig. 5) correlates very well with inhibition of the alternative pathway of human C by cysteine (Takada et al., 1978). In analogy with human and guinea pig alternative C activation, RRBC membrane-bound murine C_{3b} and alternative pathway C_3 convertase are not very sensitive to the regulatory influence of the potent murine C_{3b} inactivator system (Platts-Mills and Ishizaka, 1974; Czop et al., 1978; Ranken and Linscott, 1979).

The AP50 activity of mouse serum measured with RRBC as indicator cells is of the same order as that of human and guinea pig serum (Fig. 1). With MRBC as indicator cells, however, human AP50 activity is 1.5 times higher, while mouse and guinea pig serum have no measurable activity. This discrepancy suggests that besides sialic acid (Kazatchkine et al., 1979) other membrane components govern the inhibition of alternative C pathway activation by erythrocytes. Experiments to investigate the possible use of a combination of human serum and mouse erythrocytes as a tool for quantitation of the murine C_{3b} inactivator system are in progress.

The fact that mouse serum may be used to prepare an EAC reagent functional in a C_3 rosette test (Bianco et al., 1970) indicates that mouse C is activated at least to C_3. This, with our observations of alternative pathway and terminal stage (C_5—C_9) activation of mouse C by RRBC suggest that the mouse has an intact C system. Our results underline, therefore, the conclusion of Ranken and Linscott (1979) that the failure of mouse serum to lyse EA is due to the activity of C_{3b} inactivator. Experiments are being performed to explore the possible use of sensitised RRBC for quantification of mouse classical C pathway activity.

The technique for determining AP50 activity in mouse serum as described here shows some but no predominant sensitivity to batch variation and ageing of RRBC (Table 1; Fig. 2). In general it was better to use RRBC not older than 5 days. The results of absorption experiments with RRBC (Fig. 3) make it probable that antibodies are responsible for a Ca^{2+}-dependent lysis of RRBC by mouse serum. The slight decrease in AP50 activity as a consequence of absorption is concordant with a certain IgG dependence of AP50 activity observed for human serum by Nelson and Ruddy (1979). The optimal incubation time for measuring AP50 activity in mouse serum appeared to be 45′ (Fig. 4), but 30′ was preferred because of the lower background lysis.

Mouse serum AP50 activity is stable to incubation at temperatures up to 37°C (Fig. 6). At higher temperatures, however, complete decomplementation occurs quickly. The observation that the AP50 activity of the serum of male mice is higher than that of females (Fig. 7) agrees with observations concerning the classical C pathway of mice (Berden et al., 1978). That plasma AP50 values are substantially lower than serum values might be explained by the recent suggestion of Davis et al. (1978) that properdin factor $\overline{D}$ may be a fragment of thrombin. Inter-strain differences with respect to C activity (Table 2) have also been reported for the classical pathway (Berden et al., 1978). The higher AP50 value for C3H/HeJ serum compared with that of C3HeB/FeJ serum is in line with our hypothesis that the failure of C3H/HeJ serum to generate functional EAC complexes (Hoffmann, 1978) might be due to an aberrant C_{3b} inactivator system (Van Dijk et al., 1980).

It is concluded that the method described here is useful for determining alternative C pathway activity in mouse serum.

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