SHORT COMMUNICATIONS

Restricted Rosette Formation with an EAC Reagent Prepared with Serum of C3H/HeJ Mice

HANS VAN DIJK, DICKY VAN HEUVEN-NOLSEN, PIETERNEL M. RADEMAKER,
NANNE BLOKSMA, AND JAN M. WILJERS

*State University of Utrecht, Laboratory of Microbiology, Catharijnesingel 59,
3511 GG Utrecht, The Netherlands*

Received August 30, 1979

The EAC generating capacities of fresh and stored BALB/c, Swiss, C3HeB/FeJ, and C3H/HeJ mouse sera were investigated in a rosette test using spleen cells of each of the serum donors as indicator cells. With BALB/c spleen cells a decreased ability of both stored and fresh C3H/HeJ serum to generate functional EAC could be registered, whereas with C3HeB/FeJ spleen cells such was the case only with stored serum. Swiss spleen cells did not discriminate between the capacities of the four sera and with C3H spleen cells both C3H sera tended to be less effective than BALB/c and Swiss serum. This indicates that C3H/HeJ serum is able to generate EAC but with a somewhat different disposition of cell-bound C molecules. Our results suggest that the "defect" in C3H/HeJ serum is localized in C₄, C₃, or rather in some C-associated inhibiting system as C₄-binding protein or the C_{3b} inactivating system. A possible relationship of the "defective" serum factor and the defective Lps gene product is suggested.

INTRODUCTION

Membraneous C₃ and C₄ receptors are characteristics of B lymphocytes of different species. The presence of these receptors on cells can be visualized by rosette formation with complement (C)-coated erythrocytes (1). For the preparation of such a reagent and to prevent rosette formation via receptors for the F_c portion of IgG, sheep erythrocytes (E) are complexed with the IgM fraction of rabbit anti-E serum (A) and subsequently incubated with mouse serum as a source of nonlytic C, which results in the formation of the EAC reagent (1).

In a recent paper Hoffmann (2) described how serum of lipopolysaccharide (LPS) nonresponder C3H/HeJ mice did not support the formation of functional EAC reagents, which was ascribed to a defective or missing thermostable (30 min, 56°C) C component. In this paper it is suggested that the defective rosette formation with EAC prepared with C3H/HeJ serum is due to a modification in erythrocyte-bound C₃ or C₄, which prevents rosette formation with lymphocytes of some but certainly not all mouse strains.

MATERIALS AND METHODS

Mice. BALB/c and Swiss inbred mice were bred and maintained in the Laboratory of Microbiology, Utrecht, The Netherlands. As a rule male mice of 8-12

weeks of age were used. Male C3HeB/FeJ and C3H/HeJ mice were obtained from Jackson Laboratories (Bar Harbor, Maine). For the preparation of serum mice were bled from the orbital plexus by means of capillary tubes. Fresh serum and serum stored at -70°C immediately after harvesting were used.

Sheep erythrocytes. Sheep blood stored in Alsever's old solution was obtained from the National Institute of Public Health (RIV, Bilthoven, The Netherlands). Commercial preparations of sheep blood were reported to be incidentally contaminated with considerable numbers of EAC rosetting lymphocytes of sheep origin, which could falsely enhance the numbers of rosettes recorded (3). Therefore we routinely liberated sheep blood from leukocytes by the following procedure (4): The cells were spun for 30 min at 200g over Ficoll-Isopaque (SD 1.114), which was prepared by mixing 1 vol of Isopaque 440 (Hyegaard and Co., Oslo, Sweden) with 4.0 vol of 9% (w/v) Ficoll (Pharmacia, Uppsala, Sweden). The sedimented red cells (E) were washed three times with Veronal-saline buffer, pH 7.35 ± 0.05 , containing 0.15 mM Ca^{2+} and 0.5 mM Mg^{2+} (VSB⁺⁺; (5)), and used for further experiments.

Preparation of the IgM fraction of rabbit anti-E serum. Rabbit anti-E hemolysin was obtained from the National Institute of Public Health (RIV, Bilthoven, The Netherlands) and fractionated by ultrafiltration using Amicon filters (Amicon, Lexington, Mass.) and VSB⁺⁺ as diluent. The residue on the XM 300 filter (exclusion limit 300,000 MW) was eventually diluted to its original volume with VSB⁺⁺ and designated A.

Preparation of EAC. E and A were mixed to obtain final concentrations of 10^9 E and 1.25 μl A/ml VSB⁺⁺. After incubation for 30 min at 37°C the cells were spun down, washed once with VSB⁺⁺, and resuspended to a concentration of 10^9 cells/ml. One volume of these cells was mixed with an equal amount of VSB⁺⁺ or mouse serum diluted 1/10, 1/30, or 1/100 in VSB⁺⁺ and incubated for another 30 min at 37°C . The cells were centrifugated and resuspended in Eagle's essential medium.

EAC rosette test. The EAC rosette test was performed according to Bianco *et al.* (1). In brief, a suspension of mouse spleen cells was prepared as described elsewhere (6) and mixed with the EAC reagent so that final concentrations of 10^6 nucleated spleen cells and 5×10^7 EAC/ml were reached. After incubation for 30 min at 37°C the mixture was placed on ice and the rosettes were counted. The moderate numbers of rosettes recorded by us as compared with other authors (2, 3, 7) are to be ascribed to the purification procedure of the sheep erythrocytes and to the relatively low splenocyte/EAC ratio (3, 7) and cell concentrations (7) used rather than to the age of the spleen cell donors (8).

Statistical analysis. Results have been expressed as the mean of two independent observations of each of at least three animals $\pm$ standard error of the mean ($M \pm \text{SEM}$). For significance analysis Student's *t* test was performed. *P* values exceeding 0.05 were considered not significant (N.S.).

RESULTS

Fresh BALB/c, Swiss, C3HeB/FeJ, and C3H/HeJ sera as such or after storage at -70°C for 14 days were used to generate EAC. The EAC complexes were tested for the presence of cell-bound C_3 and/or C_4 by rosetting the cells with spleen cells of BALB/c, Swiss, C3HeB/FeJ, and C3H/HeJ mice. Figure 1 shows that BALB/c

FIG. 1. Functional EAC generating capacities of stored (●) and fresh (○) mouse sera as detected by rosette formation with BALB/c spleen cells. The spleen cell donors were 8 weeks of age. The 10% value of fresh and stored C3H/HeJ serum is significantly below that of the other sera, except for the value of stored C3HeB/FeJ serum ($P < 0.05$, two-sided). The values of 3% C3H/HeJ serum are significantly lower than those of C3HeB/FeJ serum ($P < 0.05$).

spleen cells discriminated between the EAC generating capacities of the four sera tested. Swiss and C3HeB/FeJ sera were the most potent, and BALB/c serum had an intermediate activity, while C3H/HeJ serum, although not negative, was the least effective. No obvious differences in the efficacies of fresh and stored sera were recorded.

When Swiss spleen cells were used no significant differences between the EAC generating capacities of the four sera or between the fresh and stored sera were observed (Fig. 2). Spleen cells of both C3H strains, however, differentiated between C3H sera and BALB/c and Swiss sera, as far as fresh sera were concerned (Figs. 3 and 4): The fresh C3H sera were less potent generators of EAC than were fresh BALB/c and Swiss sera. C3HeB/FeJ spleen cells, unlike those of C3H/HeJ

FIG. 2. Functional EAC generating capacities of stored (●) and fresh (○) mouse sera as tested with Swiss spleen cells. The spleen cell donors were about 10 weeks old. The 10% value of stored C3HeB/FeJ serum is significantly higher than that of BALB/c serum, and the same holds for the 10% value of fresh Swiss serum compared to C3H/HeJ serum ($P < 0.05$).

FIG. 3. Functional EAC generating potencies of stored (●) and fresh (○) mouse sera as tested with C3HeB/FeJ spleen cells. The spleen cell donors were about 3 months old. The 10% values for stored BALB/c, Swiss, and C3H/HeJ serum and the 3% value for stored Swiss serum are significantly decreased in relation to the same values for fresh serum ($P < 0.01$, $P < 0.01$, $P < 0.05$, and $P < 0.025$, respectively). All values of stored C3H/HeJ serum do not significantly differ from zero.

origin, discriminated moreover between fresh and stored serum (Fig. 3). In this instance stored sera were far less efficient in generating EAC, with the exception of stored C3HeB/FeJ serum. Stored C3H/HeJ serum failed to generate functional EAC as tested with C3HeB/FeJ spleen cells.

DISCUSSION

In a recent paper Hoffmann (2) described the (in)ability of different mouse sera to generate EAC reagents functional in a rosette test with C3HeB/FeJ spleen cells. He observed that serum of LPS nonresponder C3H/HeJ mice, unlike sera of, e.g., BALB/c, Swiss inbred, or C3HeB/FeJ mice, was not able to form a functional EAC reagent. The defect in the C3H/HeJ serum could be reversed by a thermostable (30 min, 56°C), LPS-sensitive serum factor from C3HeB/FeJ mice.

In the present study we repeated the experiments of Hoffmann mentioned and investigated moreover the ability of EAC prepared with C3H/HeJ serum to form rosettes with spleen cells of mouse strains other than C3HeB/FeJ. We compared the numbers of rosettes formed with EAC prepared from C3H/HeJ serum with those formed with EAC from BALB/c, Swiss, and C3HeB/FeJ serum using spleen cell suspensions of BALB/c, Swiss, C3HeB/FeJ, or C3H/HeJ origin. In contrast with Hoffmann (2), who used stored sera only, we consequently used both fresh and stored mouse serum to generate EAC.

Our experiments indicate that the EAC generating capacities of the four sera tested are exponents of the serum storage procedure and the origin of the rosetting spleen cells. The failure of stored C3H/HeJ serum to generate functional EAC as tested with C3HeB/FeJ spleen cells (Fig. 3) was as reported by Hoffman (2). Our findings add to this, however, that the defect observed is acquired during the storage procedure and that C3HeB/FeJ spleen cells are the only cells that discriminate between fresh and stored sera. Further it is shown that BALB/c and C3H, but not Swiss spleen cells, discriminate between the relative efficacies of

the four sera to generate EAC. When BALB/c spleen cells are used, C3H/HeJ serum seems to be the least efficient (Fig. 1), whereas with C3H spleen cells both C3H sera seem less efficacious than BALB/c and Swiss serum (Figs. 3 and 4).

At variance with those of Hoffmann (2) our observations imply that fresh, but also stored C3H/HeJ serum gives rise to functional EAC (Figs. 2 and 4). The cell-bound C components seem, however, to be present in a form which does not permit recognition by C3HeB/FeJ spleen cells, as far as stored serum is concerned, and which is less readily recovered by BALB/c splenocytes. As not only receptors for the C_{3b} portion, but also for C_{3d} (9) and C_{4b} seem to be involved in EAC rosette formation our results can be explained by a differential exposition of C_{3b} , C_{3d} , and C_{4b} receptors on the lymphocyte surfaces of the different mouse strains.

The presence of functional C on EAC generated with C3H/HeJ serum makes it improbable that the serum "defect" is localized in the early classical C system preceding C_3 . The thermostability of the "defective" factor excludes the possibility that it is factor B or D of the alternative C pathway, leaving the possibilities that it could be C_3 itself or factor P. In contrast to this, however, other reports mention that the level of C_3 in C3H/HeJ mice is quite normal (2) and that the hemolytic titer involving the whole C cascade is similar to that of C3HeB/FeJ mice (10) or even enhanced (11). Therefore the "defect" would rather be localized in some C-associated inhibitory system as C_4 -binding protein (C_4 -bp (12)) or the C_{3b} inactivating system in other animal species consisting of C_{3b} inactivator (C_{3b} INA) and β_1H (13, 14). In that formation the reconstitution experiments of Hoffmann (2) with heat-inactivated C3HeB/FeJ serum suggest the restoring factor to be C_4 , C_3 , or a factor of the C_{3b} inactivator system. C_4 would give rise to more C_{4b} and indirectly more C_{3b} and/or C_{3d} molecules on the EAC surface. C_3 would generate more erythrocyte-bound C_{3b} and indirectly C_{3d} , while the C_{3b} inactivator system

FIG. 4. Functional EAC generating efficacies of stored (●) and fresh (○) mouse sera as tested with C3H/HeJ spleen cells. The spleen cell donors were about 3 months of age. Although the values of both C3H sera tend to be lower than those of BALB/c and Swiss serum, none of the differences are significant, with the exception of for 3% stored C3HeB/FeJ versus C3H/HeJ serum ($P < 0.05$).

would promote C_{3d} expression on the EAC cells. The thermostability as considered for C_1 and C_3 of other animal species and as recently demonstrated for the murine C_{3b} inactivator system (15) is in line with the idea that the restoring factor would be one of these three.

Whatever the identities of the "defective" factor of C3H/HeJ serum and of the restoring factor of C3HeB/FeJ serum might be, it would be interesting to know its (their) relation to the LPS gene product (16–18) expressed as LPS receptor (19, 20) on B cells and macrophages (18) and to products of macrophages stimulated with LPS, such as glucocorticoid antagonizing factor (21) and tumor necrosis factor (23); the more so, in the context of this paper, because LPS is a potent activator of both the classical and the alternative C pathways (23, 24). Further study will therefore be directed toward a possible relationship between the "defective" serum factor of C3H/HeJ mice and the factors referred to.

REFERENCES

1. Bianco, C., Patrick, R., and Nussenzweig, V., *J. Exp. Med.* **132**, 702, 1970.
2. Hoffmann, M. K., *J. Immunol.* **121**, 619, 1978.
3. O'Neill, P. A., Woodson, D. L., and Mackler, B. F., *J. Immunol.* **121**, 2586, 1978.
4. Ferrante, A., and Thong, Y. H., *J. Immunol. Methods* **24**, 389, 1978.
5. Mayer, M. M., In "Experimental Immunochemistry" (E. A. Kabat and M. M. Mayer, Eds.), p. 133. Charles C Thomas, Springfield, Ill., 1961.
6. Kerckhaert, J. A. M., Benner, R., and Willers, J. M. N., *Immunology* **25**, 103, 1973.
7. Dierich, M. P., Pellegrino, M. A., Ferrone, S., and Reisfeld, R. A., *J. Immunol.* **112**, 1766, 1974.
8. Gelfand, M. C., Elfenbein, G. J., Frank, M. M., and Paul, W. E., *J. Exp. Med.* **139**, 1125, 1974.
9. Ranken, R., and Linscott, W. D., *J. Immunol.* **120**, 1793, 1978.
10. Moeller, G. R., Terry, L., and Snyderman, R., *J. Immunol.* **120**, 116, 1978.
11. Sulzer, B. M., and Goodman, G. W., In "Microbiology 1977" (D. Schlessinger, Ed.), p. 2304. Amer. Soc. Microbiol., Washington, D.C., 1977.
12. Ferreira, A., Takahashi, M., and Nussenzweig, V., *J. Exp. Med.* **146**, 1001, 1977.
13. Harrison, R. A., and Lachmann, P. J., *J. Immunol.* **120**, 1777, 1978.
14. Ruddy, S., Carlo, J., and Conrad, D., *J. Immunol.* **120**, 1794, 1978.
15. Ranken, R., and Linscott, W. D., *J. Immunol. Methods* **26**, 283, 1979.
16. Watson, J., and Riblet, R., *J. Exp. Med.* **140**, 1147, 1974.
17. Ruco, L. P., Meltzer, M. S., and Rosenstreich, D. L., *J. Immunol.* **121**, 543, 1978.
18. Rosenstreich, D. L., Vogel, S. N., Jacques, A. R., Wahl, L. M., and Oppenheim, J. J., *J. Immunol.* **121**, 1664, 1978.
19. Haas, G. P., Johnson, A. G., and Nowotny, A., *J. Exp. Med.* **148**, 1081, 1978.
20. Wahl, L. M., Rosenstreich, D. L., Glode, H., Sandberg, A. I., and Mergenhagen, S. E., *Infect. Immunity* **23**, 8, 1979.
21. Moore, R. N., Goodrum, K. J., Couch, R. E., and Berry, L. J., *Infect. Immunity* **19**, 79, 1978.
22. Koenig, S., Hoffman, M. K., and Thomas, L., *J. Immunol.* **118**, 1910, 1977.
23. Fearon, D. T., and Austen, K. F., In "Immunochemistry: An Advanced Textbook" (L. E. Glynn and M. W. Steward, Eds.), p. 365. Wiley, Chichester, 1977.
24. Galanos, C., *Z. Immunitätsforsch.* **149**, 214, 1975.