PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 151, 526-531 (1976)

Evidence for Clustering of H-2K, H-2D, and the Fc Receptor on the Membranes of B Cells[1]

M. L. TYAN

Dental Research Center, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27514

B lymphocytes[2] and macrophages from several species including man and the mouse have been shown to bear on their membranes a trypsin-resistant protein distinct from surface Ig and the C3 receptor that specifically binds the Fc portion of heat-aggregated or antigen-complexed immunoglobulin (1–5). This Fc receptor has specificity for a site on the C_H3 region of Ig (6), and the integrity of the site is dependent on intact disulfide bonds (5). Of the various immunoglobulin classes, IgG interacts best with this receptor, and its binding by B cells is not dependent on temperature, pH, divalent cations, or complement (3–5).

Dickler and Sachs (7) have shown that the binding of fluorescein-labeled aggregated IgG to mouse B cells can be markedly inhibited by pretreatment of the lymphocytes with antisera to the H-2 complex. The antibodies responsible for the inhibition appeared to be specific for alloantigens expressed primarily on B cells and determined by the Ir region of the H-2 complex (Ia antigens) (8, 9). In their studies, antisera to Ia antigens produced inhibition of binding, whereas antisera specific for mouse Ig, H-2K, and H-2D did not. On the basis of these and other experiments, they concluded that the Fc receptor and certain of a series of alloantigens determined by the Ir region of the H-2 complex are identical or closely associated on the membranes of B cells.

Presented below are observations that support this concept. In addition, these studies suggest that H-2K and H-2D may be situated in close proximity to the Fc receptor on the membrane of B cells and, together with the β_2-microglobulin associated with H-2 specificities on T cells, they may form a functional unit critical to B cell–T cell interactions.

Materials and methods. Mice. Adult male CBA/J, C57BL/6, $C3D2F_1$, and $B6D2F_1$ mice were purchased from Jackson Laboratories, Bar Harbor, Maine. B10.AQR and B10.6R mice were bred in this laboratory; the original breeding pairs were generously donated by Dr. D. C. Shreffler, Department of Human Genetics, University of Michigan School of Medicine. The H-2 haplotypes of these mice are listed in Table I.

Antisera. C57BL/6 anti-CBA/J, CBA/J anti-C57BL/6, $C3D2F_1$ anti-B10.AQR, and B10.6R anti-B10.AQR sera were produced by multiple injections of washed spleen cells as described previously (5). The anti-θ serum was produced by repeatedly injecting AKR mice with C3H/HeJ thymocytes. In certain experiments C57BL/6 anti-CBA and B10.6R anti-B10.AQR sera were used after they had been absorbed four times with equal volumes of washed donor spleen or thymus cells. (B10 × LP.RIII) F_1 anti-B10.A (5R) and (B10.AKM × C3H.SW) F_1 anti-B10.A sera were obtained from the Jackson Laboratories. All antisera and normal sera were heat-inactivated at 56° for 20 min. The predicted interactions of the antisera with specific cells are outlined in Table I; they were used at a final dilution of 1:100 unless otherwise indicated. RAMIG (Miles Laboratories, Kankakee, Illinois) was absorbed three times with washed mouse thymocytes and used 1:1000.

Aggregated proteins. Human IgG, albu-

[1] This work was supported by USPHS Research Grants Nos. HD-7708, DE 02668, and RR 5333.

[2] Abbreviations used in this paper: B lymphocyte, bursa-derived cell; T cell, thymus-derived cell; Ig, immunoglobulin; C3 receptor, membrane-binding site for complex of antigen–antibody-complement; FITC, fluorescein isothiocyanate conjugated; Ir, immune response gene 1; Ia, Ir-region associated; H-2 complex map, (K, Ir, Ss, D); SRBC, sheep red blood cells; EA, 7 S rabbit anti-SRBC antibody-coated SRBC; EAC, 19 S rabbit anti-SRBC antibody-coated SRBC reacted with mouse complement; BSA, bovine serum albumin; PBS, phosphate-buffered saline, pH 7.2; RAMIG, rabbit anti-mouse IgG; β_2-μ, β_2-microglobulin.

Copyright © 1976 by the Society for Experimental Biology and Medicine
All rights reserved.

min, FITC-IgG, and FITC-albumin (Miles Laboratories, Kankakee, Illinois) were heat-aggregated at 63° for 20 min (5), and they were used at a concentration of 100 μg/ml.

β_2-microglobulin and Fc-IGG. The Fc portion of human IgG was purchased from Worthington Biochemical Corp., Freehold, N.J. Human β_2-microglobulin was generously supplied by Dr. M. D. Poulik, Wayne State School of Medicine, Detroit, Michigan.

FITC-protein binding studies. The binding of FITC-proteins to lymphocytes was performed as described by Dickler and Sachs (7). The degree of binding was determined by fluorescent microscopy on fixed preparations and on single cells in suspension by the Cytofluorograph, Bio/Physics Systems, Mahopac, N.Y. At least 500 cells per group were examined by the former method, and 5 × 10^5 cells were scanned and the intensity of fluorescence determined by the latter.

EA and EAC rosette formation. The formation and enumeration of spleen lymphocyte EA and EAC rosettes were performed by standard methods (10, 11). Briefly, spleen cells were washed three times, suspended at a concentration of 25 × 10^6/ml in PBS, PBS with 0.01% Na azide, or in PBS with 1% BSA with or without Na azide. The cells were incubated with antiserum at room temperature or 4° for 30–45 min, and they were washed three times prior to the addition of 1% EA or EAC. In capping experiments (12), RAMIG was added after the first series of washes, and the cells were incubated for 2 hr at room temperature or 4° with or without Na azide to prevent capping. The cells were washed three times and EA or EAC added as above. The cell suspensions were examined in a hemocytometer and a minimum of 1000 lymphocytes were counted. EA and EAC were made with subagglutinating amounts of 7 S and 19 S rabbit anti-SRBC sera, respectively (Cordis Laboratories, Miami, Florida), and fresh mouse serum (1:10) was the source of complement for EAC. Inhibition of rosette formation was determined by comparing the number of rosettes formed by cells incubated with normal mouse serum or AKR anti-θC3H serum with the number formed by cells incubated with the test serum.

Results. The effects of antisera to H-2 complex components on EA and EAC ro-

TABLE I. INHIBITION OF EA ROSETTE FORMATION BY ALLOANTISERA DIRECTED AGAINST H-2K, H-2D, AND IR SPECIFICITIES[a].

	Percentage inhibition (± S.D.)[b]				
	Source of spleen cells (H-2 complex genotype)[c] and predicted interaction with antisera				
Antiserum (1:50)	CBA/J (k, k, k, k)	C57BL/6 (b, b, b, b)	B10.AQR (q, k, d, d)	B10. 6R (q, q, q, d)	B6D2F_1 (bd, bd, bd, bd)
C57BL/6 anti-CBA/J	k, k, k, k	– – – –	11, Ir–3	11– –3	– – –3
	86 ± 2.3	0	89 ± 4.6	63 ± 7.2	66 ± 6.5
CBA/J anti-C57BL/6	– – – –	b, b, b, b	– – –6, 27-29[d]	– –6, 27-29[d]	b, b, b, b
	8.2 ± 2.1	97 ± 1.4	88 ± 6.2	94 ± 1.4	96 ± 1.6
(B10 × LP.RIII)F_1	– – – –	– – – –	– – –4, 13	– – –4, 13	– – –4, 13
anti-B10.A (5R)	0	5.1 ± 1.7	91 ± 4.3	86 ± 1.1	92 ± 2.3
C3D2F_1 anti-B10.AQR	– – – –	– – – –[d]	17 – – –	17 – – –	– – – –
	0	6.2 ± 1.4	83 ± 4.2	92 ± 3.4	0
B10.6R anti-B10.AQR	– Ir – –	– – – –	– Ir, Ss –	– – – –	– – Ss –
	58 ± 6.5	0	70 ± 5.7	0	0
(B10.AKM X C3H. SW)F_1	– – – –	– – – –	– – Ss, 4	– – – 4	– – Ss, 4
anti-B10.A	6.3 ± 2.4	5.8 ± 1.2	47 ± 1.5	42 ± 4.0	49 ± 2.1

[a] These data were compiled from 32 separate experiments.
[b] The number of rosettes obtained in AKR anti-θC3H treated samples was used as the 100% value.
[c] (H-2K, Ir, Ss, H-2D).
[d] This antiserum could conceivably contain antibodies to IgG_{2a} of the target cell allotype.

sette formation were evaluated in four strains of homozygous mice and in one F_1 hybrid (Table 1). In agreement with the results obtained when FITC-IgG was used as the ligand for the Fc receptor (7) it was found that: (a) anti-Ia sera inhibited EA rosette formation by cells bearing that Ir product (B10.6R anti-B10.AQR sera with CBA/J and B10.AQR cells), (b) anti-H-2 sera raised against a different H-2 haplotype inhibited EA binding by cells that share Ia antigens (e.g., C57BL/6 anti-CBA/J sera with B10.AQR cells), and (c) absorption of sera containing antibodies to Ia antigens with T cells of the donor type failed to diminish their inhibitory properties (e.g., C57BL/6 anti-CBA/J and B10.6R anti-B10.AQR sera absorbed 4× with donor thymus cells did not lose the ability to inhibit EA rosette formation by CBA/J and B10.AQR cells; however, adsorption with donor spleen cells removed all inhibitory activity). None of these antisera by themselves had significant effects on EAC rosette formation.

However, in contrast with previous studies (7), it was found that anti-H-2 complex sera that reacted with H-2K or H-2D but not Ia specificities were equally able to inhibit EA rosette formation: as examples, (a) (B10 × LP.RIII)F_1 anti-B10A (5R) with B10.AQR, B10.6R, and B6D2F_1 cells, (b) C3D2F_1 anti-B10.AQR with B10.AQR and B10.6R cells, and (c) (B10.AKM × C3H.SW)F_1 anti-B10.A with B10.AQR, B10.6R, and B6D2F_1 cells. In addition, antisera reacting with H-2 specificities of one parental allotype produced a profound rather than the expected partial inhibition of EA rosette formation by cells from F_1 hybrid mice (B6D2F_1) where H-2 and Ia products of both parental types have been shown to be expressed on every B lymphocyte (7).

In view of evidence that Fc receptors (2) as well as H-2 (13) and HL-A (14) products can be found in discrete patches on cell surfaces, these results suggested among other possibilities, that Ia and H-2 may be clustered in such a manner that antibodies reacting with H-2 specificities may block most Fc receptors by means of steric hindrance or by binding of the Fc portion of the attached antibody to an adjacent Fc receptor. To test this hypothesis, B10.6R and B6D2F_1 cells were incubated with antisera that reacted with H-2K or H-2D, and then a rabbit anti-mouse IgG serum was added under conditions that would or would not (Na azide added) allow capping of these products. In Table II, data are presented that show that after capping of the H-2 products had been allowed to occur there was a significant increase in EA rosette formation, but where capping had been prevented, rosette formation remained profoundly inhibited. This was true for cells from heterozygous as well as homozygous donors, and similar results were obtained when capping was prevented by keeping the cells at 4°. No changes were noted under these experimental conditions when the Fc receptor itself was blocked (e.g., aggregated IgG; B10.6R anti-B10.AQR with B10.AQR cells, data not shown; CBA anti-C57BL/6 with B6D2F_1 cells).

These results suggested that the inhibition of EA rosette formation by antisera to H-2D and H-2K was the result of the close proximity of these products to the Fc receptor, and that the apparent conflict between these studies and those using FITC-IgG (7) was due to differences in size and/or charge of the ligands. To test this, aliquots of cells treated with antisera to H-2K or H-2D were reacted with EA or FITC-IgG. In Table III it can be seen that under identical experimental conditions EA rosette formation was inhibited by antisera to H-2K and H-2D but the binding of FITC-IgG as measured microscopically and electronically was not. On the other hand, masking the Fc receptor itself with aggregated IgG impaired the binding of both.

Because β_2-microglobulin has a striking homology to the Fc portion of IgG (15), the interactions of β_2-μ with the Fc receptor on mouse B cells were studied (see ref. 16 for reactions with macrophages). It was found that at high concentrations β_2-μ and the Fc portion of human IgG (aggregated and unaggregated) moderately inhibited EA rosette formation (Fig. 1). More striking, however, was the observation that at lower concentrations β_2-μ and to some degree the reduced and alkylated Fc portion of IgG produced significant increases in EA bind-

TABLE II. EFFECTS OF CAPPING ON THE INHIBITION OF EA ROSETTE FORMATION PRODUCED BY ALLOANTISERA TO H-2 COMPLEX SPECIFICITIES OF B10.6R AND B6D2F$_1$ SPLEEN CELLS.[a]

	Percentage inhibition							
	B10.6R				B6D2F$_1$			
	PBS		PBS + 0.01% Na azide		PBS		PBS + 0.01% Na azide	
Agent	EA	EAC	EA	EAC	EA	EAC	EA	EAC
Mouse serum	8	0	4	0	6	0	7	0
AKr anti-θC3H serum[b]	12	4	16	6	21	0	16	0
Aggregated IgG[c]	89	0	81	0	79	0	76	0
C3D2F$_1$ anti-B10.AQR[d]	23	4	71	0	0	0	0	0
(B10 × LP.RIII)F$_1$[e] anti-B10.A(5R)	13	0	67	0	24	14	89	10
CBA anti-C57BL/6[f]					84	12	94	16
C57BL/6 anti-CBA[g]					0	0	68	0

[a] After incubation for 45 min with the above agents the spleen cells were washed three times, and RAMIG (1:1000) was added in an effort to produce capping of the membrane component bound by the first agent. Sodium azide inhibits capping.
[b] Reacts with T cells.
[c] Reacts with all F_c receptors.
[d] Reacts with H-2K of B10.6R but not B6D2F$_1$.
[e] Reacts with H-2D product of B10.6R and B6D2F$_1$.
[f] Reacts with entire H-2 complex. See footnote [b].
[g] Reacts with H-2D.

TABLE III. INHIBITION OF EA BUT NOT AGGREGATED IgG BINDING BY ANTISERA TO K AND D SPECIFICITIES OF B10.6R MICE .[a]

		Percentage inhibition		
			Fluorescence	
Agent	FITC Agent	EA Rosettes	Electronically	Microscopically
Mouse serum	IgG	0	0	0
AKr anti-θC3H	IgG	30	−4	0
Aggregated IgG	IgG	96	40	65
C3D2F$_1$ anti-B10.AQR	IgG	80	−9	6
(B10.AKM × C3H.SW)F$_1$ anti-B10.A	IgG	91	−23	−5
AKr anti-θC3H	Albumin		N.S.[b]	N.S.

[a] Spleen cells were incubated with the antisera as described. After 30 min they were washed three times, and either EA or FITC aggregated IgG was added to the divided samples. Cells were incubated with FITC IgG for 30 min, washed three times, and binding of IgG to the cells was determined by fluorescent microscopy and electronically (Cytofluorograph, Bio/Physics Systems, Mahopac, N. Y.).
[b] No significant binding.

ing; that is, more cells bound EA and individual rosettes were generally much larger. Finally, it was found that tanned SRBC coated with β_2-μ were bound by mouse spleen cells, and this could be inhibited by aggregated IgG or enhanced by low concentrations of β_2-μ or monomeric Fc fragments (data not shown).

Discussion. Using specific alloantisera to inhibit the binding of FITC-IgG by the Fc receptor, Dickler and Sachs in their first report (7) presented evidence indicating that the Fc receptor and certain Ia antigens are either identical or closely associated on the membranes of B lymphocytes. More recently, they reported that antigens determined by at least one non-H-2 locus also are associated with or are a part of Fc receptors (17).

Although the experiments reported here tend to support the concept of Fc/Ia identity, it was also found that antisera to H-2K and

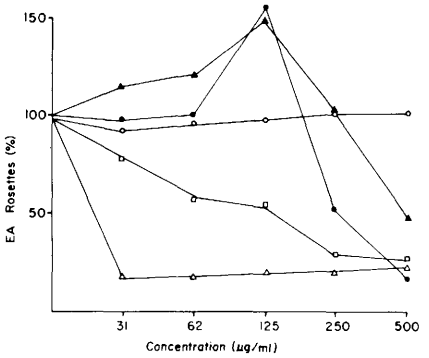

FIG. 1. Inhibition of EA rosette formation by aggregated IgG (△), aggregated albumin (○), aggregated F_c fragments of IgG (□), β_2-microglobulin (▲), and the reduced and alkylated F_c fragments of IgG (●). The data are representative of the results obtained in four experiments.

H-2D were as effective as anti-Ia sera in inhibiting the binding of ligand (EA) by the Fc receptor. This apparent conflict seems to have been resolved by the following observations: (a) when capping of H-2 products was allowed to occur EA rosette formation was only minimally impaired, (b) when aliquots of cells treated with antisera to H-2K or H-2D were reacted with EA or FITC-IgG, EA rosette formation was inhibited but the binding of FITC-IgG was not, and (c) when H-2K or H-2D of one parental type was blocked on F_1 hybrid cells it appeared that Fc receptor sites of both parental allotypes were made unavailable (also see ref. 5). These results suggest that under certain conditions the Fc receptor sites on B cells are clustered in patches, intermixed with and perhaps recessed relative to the H-2D and H-2K gene products. Under these circumstances, differences in size and/or charge would allow the attachment of FITC-IgG but not EA to the Fc receptor when adjacent H-2 specificities were masked by antisera. It is not known if this configuration, if confirmed, represents the natural state or whether it is the result of experimental or physiological stimulus.

Since β_2-μ closely resembles the Fc portion of IgG (15) and is an integral component of HL-A and H-2 on B and T cells (18, 19), studies were performed to determine if β_2-μ is able to interact with the Fc receptor. It was found that SRBC coated with β_2-μ were bound by the Fc receptor, and that while high concentrations of unbound β_2-μ moderately inhibited EA binding, lower concentrations enhanced rosette formation. These findings suggest that the Fc receptor on B cells and the β_2-μ associated with the histocompatibility antigens on T cells could serve to physically link these cells during the cooperative phase in the induction of antibody synthesis. Further, when circulating freely in its monomeric form (6) β_2-μ may act to modulate the availability of the Fc receptor or similar structures.

Summary. Alloantisera to H-2K, H-2D, and Ia antigens markedly inhibited the binding of EA but not FITC-IgG by the B cell Fc receptor. EA rosette formation approached normal levels when masked H-2 but not Ia specificities were allowed to cap on the membranes of B cells. β_2-μ coated SRBC were bound by the Fc receptor, and high concentrations of soluble β_2-μ were found to moderately inhibit EA rosette formation while lower concentrations enhanced binding. The data support the concept of Fc/Ia identity, and they suggest that H-2K, H-2D, and the Fc receptor may be closely grouped on the membranes of B cells. Further, these observations suggest that the β_2-microglobulin associated with H-2 could serve to link T cells with the Fc receptor of B cells during the inductive phase of antibody synthesis.

1. Basten, A., Miller, J. F. A. P., Sprent, J., and Pye, J., J. Exp. Med. **135,** 610 (1972).
2. Basten, A., Warner, N. L., and Mandel, T., J. Exp. Med. **135,** 627 (1972).
3. Dickler, H. B., and Kunkel, H. G., J. Exp. Med. **136,** 191 (1972).
4. Eden, A., Bianco, C., and Nussenzweig, V., Cell. Immunol. **7,** 459 (1973).
5. Dickler, H. B., J. Exp. Med. **140,** 508 (1974).
6. Peterson, P. A., Cunningham, B. A., Berggard, I., and Edelman, G. M., Proc. Nat. Acad. Sci. USA **69,** 1697 (1972).
7. Dickler, H. B., and Sachs, D. H., J. Exp. Med. **140,** 779 (1974).
8. Hämmerling, G. J., Deak, B. D., Mauve, G., Hämmerling, U., and McDevitt, H. O., Immunogenetics **1,** 68 (1974).
9. David, C. S., and Shreffler, D. C., Transplantation **18,** 313 (1974).
10. Bianco, D., Patrick, R., and Nussenzweig, V., J. Exp. Med. **132,** 702 (1970).
11. Möller, G., J. Exp. Med. **139,** 969 (1974).

12. Taylor, R. B., Duffus, W. P. H., Raff, M. C., and dePetris, S., Nature New Biol. **233,** 225 (1971).
13. Stackpole, C. W., Transplant. Proc. **3,** 1199 (1971).
14. Kourilsky, F. M., Silvestre, D., Neauport-Sautes, C., Dausset, J., and Levy, J. P., Transplant. Proc. **3,** 1203 (1971).
15. Smithies, O., and Poulik, M. D., Science **175,** 187 (1972).
16. Painter, R. H., Yasmeen, D., Assimeh, S. N., and Poulik, M. D., Immunol. Commun. **3,** 19 (1974).
17. Dickler, H. B., Cone, J. L., Kubicek, M. T., and Sachs, D. H., J. Exp. Med. **142,** 796 (1975).
18. Tanigaki, N., Nakamuro, K., Appella, E., Poulik, M. D., and Pressman, D., Biochem. Biophys. Res. Commun. **55,** 1234 (1973).
19. Poulik, M. D., Immunol. Commun. **2,** 403 (1973).

Received July 9, 1975. P.S.E.B.M. 1976, Vol. 151.