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Inhibition of *in Vitro* Immune Responses by Antisera to H-2 or Ir Gene Products[1] (39026)

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Reports from several laboratories indicate that antisera directed against the gene products of the major histocompatibility locus of the mouse, guinea pig and man can react with membrane components of lymphocytes and without killing them impair their *in vitro* responses to allogeneic cells or other thymus-dependent antigens (1–4). Further, studies have suggested that antibodies reacting solely with Ir-1 gene products as well as those to the entire H-2 complex are effective in depressing these responses and that these antisera are able to inhibit thymus-dependent reactions when added to culture as late as 24 hr after the responder cells have been exposed to antigen (4). The latter observations were interpreted as suggesting that anti-H-2 complex and anti-Ir sera express most if not all of their inhibitory actions on T cells after the early phases of antigen recognition and binding, and most likely during the stage of B cell–T cell or T cell–T cell cooperation (4).

Recent reports, however, suggest that at least part of the inhibitory action of anti-H-2 complex sera may be expressed on B cells via the Fc receptor. In an elegant series of experiments, Dickler and Sachs (5) showed that the binding of FITC-IgG (fluorescein isothiocyanate conjugated IgG) to mouse B cells is markedly impaired by pretreatment of the lymphocytes with anti-H-2 complex sera, and that the antibodies responsible for this inhibition are specific for alloantigens associated with the Ir region of the H-2 complex (Ia antigens). In their studies, antisera to these Ia antigens impaired binding, whereas antisera specific for the H-2K and H-2D moieties did not. On the basis of these observations, they concluded that the Fc receptor and certain of a series of alloantigens controlled by the Ir region of the H-2 complex are identical or closely associated on the membranes of B cells. Further, in view of its apparent role in antibody-dependent lymphocyte-mediated cytotoxicity (6) and because of evidence in certain cases of Ir gene control that the defect in low responders appears to be expressed at the level of the B cell (7–9), it was proposed that the Fc receptor may play a significant role in the control of the immune response (5).

Presented below are observations supporting the concept that certain Ia antigens and the B cell Fc receptor are identical or closely associated, and that anti-H-2 complex sera, at least in part, express their inhibitory properties by masking the Fc receptor or associated structures on B lymphocytes. In addition, evidence will be presented that suggests that the immune response may be inhibited *in vitro* when H-2K or H-2D are masked by alloantisera.

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 Tables II and IV.

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 (4). The anti-θ serum was produced by repeatedly injecting AKr mice with C3H/HeJ thymocytes (4). In certain experiments C57BL/6 anti-CBA serum was used after it had been absorbed four times with equal volumes of washed CBA/J spleen or thymus cells. All antisera and normal sera were heat-inactivated at 56° for 20 min. The predicted interactions of the antisera with specific responder cells are outlined in Table IV.

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Aggregated proteins. Human IgG and albumin were obtained from Miles Laboratories, Kankakee, Illinois and heat aggregated at 63° for 20 min (5).

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 and suspended at a concentration of 25 × 10^6/ml in PBS. They were incubated with the antisera at room temperature for 30 min and then they were washed three times prior to the addition of EA or EAC. The cell suspensions were examined in a hemocytometer under 400× magnification, and a minimum of 500 lymphocytes were scanned. EA and EAC were made with 7S and 19S rabbit anti-SRBC sera, respectively (Cordis Laboratories, Miami, Florida), and fresh mouse serum (1:10) was the source of complement for the EAC.

Culture conditions. Primary responses to SRBC were elicited *in vitro* under conditions described previously (12). Briefly, an underlay of washed SRBC (2%) in 1.5 ml of agar (0.25%) was placed in 35 × 10 mm tissue culture dishes (Falcon Plastics, Oxnard, California) and 4 ml of spleen cell suspension (20 × 10^6 nucleated cells) was added after the agar had hardened. Sera and mitogens (Con A, Miles Laboratories, 2 μg/ml or LPS, Difco, Detroit, MI, 5 μg/ml) were added and the cells were cultured at 37° in 5% CO_2 in air for 3 days. At this time the medium and cells were discarded and 1 ml of complement (1:10) was added to each dish. After 1.5 hr at 37° the areas of focal hemolysis were counted. All cultures were done in triplicate and replicate samples were generally within 5% of the mean.

Previous work (Tyan, unpublished data) indicates that each area of hemolysis found in the agar represents a cluster of specific antibody-forming cells. When the cells are carefully removed from the agar and cultured on SRBC-agar coated slides to detect single PFC, from 70 to 180 PFC are found for each area of hemolysis noted on the plates. The hemolysis on the plates is specific because (1) it is complment dependent, (2) cells cultured on agar without SRBC do not contain SRBC-PFC on transfer, and (3) cells cultured on SRBC-agar do not contain PFC to horse RBC or vice versa. The culture medium was Eagle's minimum essential suspension medium, augmented with nonessential amino acids, L-glutamine, 10% heat inactivated fetal calf serum, and penicillin and streptomycin (100 U and 100 μg/ml, respectively).

Results. In vitro primary responses. CBA/J and B10.AQR spleen cells cultured in NMS with SRBC for 3 days produced 28.1 and 14.6 hemolytic foci per dish, respectively (Table I); the addition of Con A or LPS to the cultures resulted in two to threefold increases. Alloantisera to the H-2^k complex or to Ir^k greatly diminished the number of foci produced and moderately impaired the enhancement induced by Con A; however, these antisera did not significantly impede the increase in foci stimulated by LPS. Absorption of the antisera with CBA spleen cells removed almost all inhibitory properties, but absorption with CBA thymocytes had no effect (relevant Ia antigens are either not expressed or are sparse on T cells (5)). In addition, these and undepicted data suggest that antisera reacting with H-2 and Ir specificities inhibit Con A stimulation of antibody-production to a greater degree than does one reacting only with Ir (e.g., C57BL/6 anti-CBA with CBA vs B10.AQR cells).

Inhibition of EA rosette formation. EA but not EAC rosette formation by spleen cells from CBA/J, B10.AQR and unexpectedly, B10.6R mice was markedly inhibited by an antiserum to the H-2^k complex (Table II). Absorption of this serum with CBA thymocytes resulted in the loss of its ability to inhibit EA rosettes by B10.6R cells; however, it remained fully capable of profoundly depressing rosette formation by cells from CBA and B10.AQR mice. Since B10.6R mice share H-2D specificity 3 but not Ir with the other two strains, these observations suggested that H-2 specificities on B cells may be associated with the FC receptor or somehow involved in EA rosette formation.

Time course studies. CBA/J spleen cells were cultured on SRBC-agar and aggregated IgG or antisera to H-2^k or Ir^k were added at time 0 or 6, 20 and 44 hr later (Table III).

TABLE I. INHIBITION OF *IN VITRO* RESPONSE TO SRBC BY ALLOANTISERA TO THE H-2^k COMPLEX OR TO Ir^k. SPLEEN CELLS WERE CULTURED ALONE, WITH CON A (2 μg/ml) OR LPS (5 μg/ml), IN NMS (1:50) OR IN ANTISERA (1:50) THAT WERE UNABSORBED OR ABSORBED (4×) WITH CBA SPLEEN OR THYMUS CELLS.[a]

			Mean number of hemolytic foci per culture Sera					
			C57BL/6 anti-CBA absorbed			B10·6R anti-B10·AQR absorbed		
Responder cells	Mitogen	NMS	Alone	With spleen	With thymus	Alone	With spleen	With thymus
CBA/J	None	28.1	2.3	26.4	3.4	5.9	29.3	6.2
	Con A	62.0	8.1	58.5	9.6	12.4	64.7	13.4
	LPS	46.6	38.3	43.2	42.5	42.3	41.9	39.8
B10·AQR	None	14.6	1.3.	13.2	1.1	4.2	15.5	3.9
	Con A	42.7	27.2	43.1	22.4	25.1	46.2	29.4
	LPS	39.8	36.5	37.4	40.1	40.7	37.8	41.5

[a] The data represent the averages of three separate experiments.

TABLE II. INHIBITION OF MOUSE SPLEEN CELL EA ROSETTE FORMATION BY ANTI-H-2 COMPLEX ALLOANTISERUM AFTER ABSORPTION WITH THYMUS CELLS.[a]

	Inhibition (%) Source of spleen cells (H-2 complex genotype)[b]					
	CBA/J (k, k, k, k)		B10·AQR (q, k, d, d)		B10·6R (q, q, q, d)	
Agent added (concentration)	EA	EAC	EA	EAC	EA	EAC
Normal mouse serum (1:50)	16	0	18	0	11	0
Akr-anti-θC3H serum (1:50)	21	0	24	0	19	0
Aggregated IgG (100 μg/ml)	97	3	94	0	98	0
Aggregated albumin (100 μg/ml)	12	4	8	2	6	3
C57BL/6 anti-CBA spleen (1:50)	85	0	93	0	72	0
C57BL/6 anti-CBA spleen absorbed 4× with CBA thymus (1:25)	72	0	91	0	0	0

[a] The data represent the averages compiled from five separate experiments.
[b] (H2K—Ir—Ss—H-2D).

The development of hemolytic foci was profoundly inhibited when these agents were added up to 20 hr after the cultures were initiated; no decrease in foci was noted when the agents were added at +44 hr.

Effect of masking H-2 specificities on the primary response. Spleen cells from four strains of mice were cultured on SRBC-agar with antisera that would react with Ir, H-2K, H-2D or all H-2 complex gene products (Table IV). It was found that all such antisera, as well as aggregated IgG, produced marked inhibition of the responses to SRBC. Con A stimulation of aggregated IgG blocked cultures was depressed only 30% on the average, and the response to LPS was essentially unchanged (Data not shown).

Discussion. Data presented previously (4) demonstrated that alloantisera directed against H-2 complex specificities were able to depress the *in vitro* responses of blood cells from homozygous and F_1 mice to thymus-dependent but not to thymus-independent antigens. The same antisera had no effect on the responses of fetal liver cells to antigens of either type. The observations suggested that the impaired responses induced by the antisera were expressed primarily through T cells and were the result of the inability of the affected T cells to augment B or T cell

cooperation in response to antigenic stimulation. However, these experiments did not make clear the extent to which impairment was expressed through H-2, Ir or other H-2 complex gene products on the cell membrane.

TABLE III. EFFECTS ON THE *IN VITRO* RESPONSE OF CBA SPLEEN CELLS TO SRBC OF ADDING ANTI-H-2 OR ANTI-IR SERA AT VARIOUS TIMES DURING THE COURSE OF THE 3-DAY CULTURE.[a]

Agent added	Concentration	Inhibition of hemolytic foci (%) Time added (hr) 0	6	20	44
NMS	1:50	20	16	7	4
C57BL/6 anti-CBA/J	1:50	82	86	65	7
B10·6R anti-B10·AQR	1:50	63	70	59	10
Aggregated IgG	200 μg/ml	95	93	87	6

[a] The data presented are from a representative experiment. Similar results were obtained in eight other experiments using CBA/J, C3H/HeJ as well as B10·AQR spleen cells.

Presented here are observations showing that immune function may be depressed *in vitro* by alloantisera to H-2D, H-2K or Ir-region specificities, and that this inhibitory action may be expressed up to 24 hr after responder cells have been exposed to antigen, i.e., after the early phases of antigen recognition and binding. The association of the inhibitory properties of these antisera and of aggregated IgG with their ability to depress EA rosette formation suggests that at least part of their effect on the immune process is made manifest by obscuring or blocking the Fc receptor on B cells. These observations tend to support the proposal that the Fc receptor may play a role in the regulation of the immune response (5).

On the other hand there is evidence that not all of the inhibitory properties of these antisera are necessarily expressed through the Fc receptor on B cells:

(a) The antisera have virtually no effect on the immune responses of pure B cell populations (4).

(b) Ia antigens have been identified on T cells (13) and although their function is unknown there is no reason at the present

TABLE IV. INHIBITION OF THE *IN VITRO* PRIMARY RESPONSE TO SRBC BY ALLOANTISERA TO H-2K, H-2D AND Ir SPECIFICITIES.[a]

Antiserum (1:50)	Inhibition (% ± SD) Source of spleen cells (H-2 complex genotype)[b] and predicted interaction with antisera: CBA/J (k, k, k, k)	C57BL/6 (b, b, b, b)	B10·AQR (q, k,d, d)	B10.6R (q, q, q, d)
AKr anti-θC3H	12 ± 6.2	5.1 ± 3.4	7.3 ± 5.6	6.4 ± 2.7
Aggregated IgG (100 μg/ml)	Fc	Fc	Fc	Fc
	82 ± 5.0	86 ± 11.2	95 ± 6.0	92 ± 7.2
C57BL/6 anti-CBA/J	k, k, k, k	— — — —	11, Ir — 3	11 — — 3
	78 ± 4.3	6.4 ± 2.1	91 ± 6.5	78 ± 4.2
CBA/J anti-C57BL/6	— — — —	b, b, b, b[c]	— — — 6, 27-29[c]	— — — 6, 27-29[c]
	7.5 ± 1.2	82 ± 4.3	68 ± 10.1	76 ± 4.4
C3D2F$_1$ anti-B10·AQR	— — — —	— — — —[c]	17 — — —	17 — — —
	4.1 ± 1.2	5.4 ± 3.3	78 ± 3.4	82 ± 6.5
B10·6R anti-B10·AQR	— Ir — —	— — — —	— Ir, Ss —	— — — —
	62 ± 5.1	4.3 ± 2.2	58 ± 6.1	5.4 ± 2.1

[a] The results are expressed as a percentage of the response obtained when spleen cells were cultured in normal mouse serum. These data were compiled from 15 separate experiments; each value represents at least four separate determinations.

[b] (H-2K, Ir, Ss, H-2D).

[c] anti-allotype (IgG$_{2a}$).

time not to believe they may be involved in regulation of the immune response.

(c) Antibodies to histocompatibility antigens (14) or to β_2-microglobulin (15, 16) can react with T cells and impair their function directly or by steric hindrance of T cell mitogen receptor sites. The data presented here lend support to this: antisera reacting with the entire H-2 complex were more suppressive than were the same sera reacting only with Ir (Ia), and the responses to Con A stimulation were more severely impaired under the former conditions.

The fact that these antisera do not depress the responses of B cell populations to antigen (4) or spleen cells to LPS suggests that B cell function per se is not directly affected by these agents. When viewed with the time course studies, these data are compatible with a construct placing the masking antibodies on H-2, Ir, Ia (Fc) and perhaps other specificities of both T and B cells, obstructing their interaction at a stage well past antigen recognition and binding.

Conclusions. Heat aggregated IgG and antisera reacting with Ir-1 products (Ia antigens) inhibited the *in vitro* primary responses of mouse spleen cells to SRBC by 50–90% and depressed EA but not EAC rosette formation by 70–90%. These experiments suggest that alloantisera to the H-2 complex express at least part of their inhibitory properties by blocking the Fc receptor on B cells, and the data support the concept that certain Ia antigens and the Fc receptor are identical or closely associated. In addition, these studies showed that the immune response may be inhibited by antisera that react with H-2K and H-2D gene products.

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